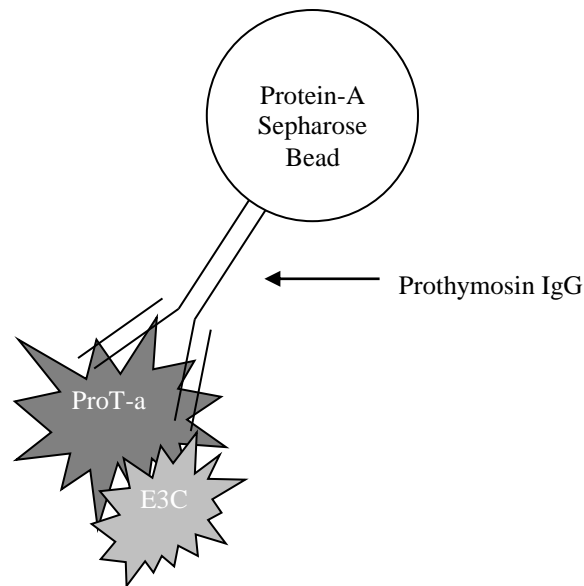


Immunoprecipitation Experiment with EBNA3C and Prothymosin- α

Protein-protein interactions are known to play crucial roles in cellular signaling pathways. To study potential protein interactions with EBNA 3C, an EBV positive B lymphocyte-derived cDNA library was screened using a yeast two-hybrid system. A truncated form of EBNA 3C (365-992aa) was cloned into a yeast expression vector and used to screen for interaction with other cellular proteins. The proteins found to interact with this region of EBNA 3C may play a vital role in the regulation of EBV latent gene expression and may provide insight into the mechanisms for induction of proliferation.

Prothymosin- α is a protein that has been pulled out of the cDNA screen. To further investigate this finding, an immunoprecipitation can be used to determine if EBNA3C and Prothymosin- α do indeed interact. Immunoprecipitations can be performed to determine if protein-protein interactions are occurring *in vivo*. This technique utilizes the specific recognition ability of antibodies to detect and bind to their perspective antigen protein. Consequently, any cellular protein that interacts with the target protein will also be pulled out. Protein A sepharose beads are used to separate the antigen-antibody complexes from other antigens. Protein A is a protein found on the surface of *Staphylococcus aureus* cells and has the ability to bind IgG antibodies. This serves as a convenient way of purification.

Schematic Diagram of Interaction:



Reagents Needed:

Cell Lines (50 Million Cells/IP):	BL41
	BL41/B958
	IB4

RIPA (Radioimmunoprecipitation assay) Buffer*:

10mM Tris, pH 7.5
1% Nonidet-P40 (nonionic detergent)
2mM Na₂EDTA
150mM NaCl

*Add 1mM PMSF (protease inhibitor) to the buffer just before use. Keep cold. Aprotinin and Pepstatin (two other protease inhibitors) can also be added [1ug/ml].

Protocol:

1. Viable cells of all three cell lines are counted using trypan blue negative staining. 50 million cells of each cell line are collected by centrifugation. The supernatant is discarded and the cell pellet dislodged by tapping the tube. Cells are washed in 25ml 1X PBS and pelleted again by centrifugation. The supernatant is discarded and the cell pellet is dislodged again by tapping. Cells are placed on ice and remain cold (on ice or at 4°C) throughout the experiment until otherwise stated.

It is important to keep the cell lysates cold. The protease inhibitor which supplement the RIPA buffer are ineffective at warmer temperatures, thus increasing the chances of proteases present in the lysates to degrade proteins of interest.

2. Cells are resuspended in 250ul RIPA buffer (including protease inhibitors) and vortexed for 30 seconds. A 12.5ul (5%) sample is taken and set aside as a cell lysate control for gel.

Make sure the cells are completely resuspended in the RIPA Buffer. Otherwise, not all of the cells will lyse.

3. The volume of the cells is brought up to 1ml with RIPA buffer, then transferred to cold 1.5ml microcentrifuge tubes. Cells are vortexed (10-15 seconds) every 15 minutes for one hour. Lysing cells remain on ice in between vortexing.
4. Cell debris is pelleted with a 15,000 RPM spin for 6 minutes at 4°C. The supernatant is transferred to a new, cold microcentrifuge tube. Ensure that all cell debris has been removed. Re-centrifugate if necessary.
5. 2ul of normal rabbit serum is added to supernatant and rotated end-over-end for 1 hour at 4°C. This is followed by the addition of 25ul protein A sepharose beads to the supernatant. Samples are rotated end-over-end at 4°C for 1 hour.

Normal rabbit serum is added to “pre-clear” the sample. Because the serum contains other antibodies, it can be used to bind antigens present in the cell lysate which may otherwise interfere with the antigen-antibody interaction of interest .

This will decrease the amount of background caused by non-specific binding that may occur and show that the interaction is specific for the two proteins.

6. Pellet beads briefly with a 15,000 RPM spin for 1 minute at 4°C. Transfer the supernatant to a new, cold microcentrifuge tube. Add 2ul of α -Prothymosin- α to the supernatant. Rotate end-over-end overnight (12-16 hours) at 4°C.

During this time, the Prothymosin- α antibody is binding to the Prothymosin protein present in the sample.

7. Add 25ul protein-A sepharose beads to the sample. Rotate end-over-end at 4°C for atleast 1 hour.

At this point, the protein-A coupled to sepharose beads will bind the Prothymosin-a antigen/antibody complex.

8. Briefly pellet beads with centrifugation at 15,000 RPM for 1 minute at 4°C. Carefully aspirate the resulting supernatant. (Do not aspirate beads!) All beads are washed by shaking 10 times vigorously there 3 times in 1ml RIPA Buffer, pelleting beads for 1 minute at 15,000 RPM at 4°C and aspirating the supernatant in between washes.

9. Beads are resuspended in 20ul of lysis buffer, heated at 95°C for 10 minutes, and resolved in the appropriate percent SDS-PAGE:

→ 8% gel for Prothymosin- α antibody IP

→ 10% gel for EBNA3C antibody IP

The loading buffer contains SDS and β -ME. These chemicals, as well as high heat, denature proteins into their polypeptide components, allowing for clear resolution in the gel. They also disassociate the protein-A sepharose bead from the bound antibody.

The differences in pH and ionic strengths between the stack and resolution gels and the electrode buffer create a discontinuous buffer system that is necessary for proper protein resolution. Make sure that the buffers and reagents are at their proper pHs.

10. Upon completion of electrophoresis, transfer the proteins to a nitrocellulose membrane using 100V constant voltage for one hour. Keep the transfer solution cold by adding an ice block into the apparatus.

11. Perform a Western Blot using:

1° α : α -EBNA3C antibody

2° α : α -Protein-A-HRP antibody

12. Detect recognized proteins with ECL reagents and expose to x-ray film.

Expected Results:

When Western Blotting for E3C:

EBNA3C Protein is expressed at high levels in the IB4 cell line, so a strong band will be seen in the 5% lysate lane around 120 kDa. The only protein to be present in the Prothymosin- α IgG lane would be Prothymosin- α (12.5kDa) (under the best of conditions) UNLESS some interaction was occurring with another protein. This is exactly what we are looking for: if a band is present at 120 kDa (the size of EBNA3C) in the prothymosin- α IgG lane, then prothymosin- α and EBNA3C must be interacting!

Note: A reciprocal version of the above described immunoprecipitation can also be performed using α -EBNA3C IgG, followed by western blot analysis with Prothymosin- α . Proteins analyzed by 10% SDS-PAGE.