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Identifying Novel Protein-Protein Interactions Using Co-Immunoprecipitation and Mass Spectroscopy

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Abstract

Proteomics has evolved from genomic science due to the convergence of advances in protein chemistry, separations, mass spectroscopy, and peptide and protein databases. Where identifying protein-protein interactions was once limited to yeast two-hybrid analyses or empirical data, protein-protein interactions can now be examined in both cells and native tissues by precipitation of the protein complex of interest. Coupling this field to receptor pharmacology has recently allowed for the identification of proteins that differentially and selectively interact with receptors and are integral to their biological effects. It is becoming increasingly apparent that receptors in neurons do not exist as singular independent units, but rather are part of large macromolecular complexes of interacting proteins. It is a primary quest of neuroscience to piece together these interactions and to characterize the regulatory signalplexes of all proteins. This unit presents co-immunoprecipitation-coupled mass spectroscopy as one way of identifying signalplex partners.

Keywords

proteomics; neuroreceptors; signalplex; receptor interactions

INTRODUCTION

It is now clear that many neuronal proteins do not exist as sole independent units, but rather are part of large macromolecular complexes of interacting proteins. The protein constituents of these “signalplexes” can be quite dynamic with respect to space and time. Elucidating the complete array of interacting proteins involved in the regulation and signaling mediated by a given target protein (usually a receptor) has become a primary goal of neurobiology. Most protein-protein interactions identified to date were found by employing either the yeast-two hybrid system or by confirming empirical data with co-localization and/or antibody studies. However, with the sequencing of the human genome and the emergence of advanced peptide-based mass spectroscopy (MS), recent studies have documented that proteomic-based approaches may be more useful for identifying proteins involved in the biological regulation of neuronal responses. In MS-based proteomics, the protein itself is used as an affinity reagent to isolate its binding partners (Fig. 5.28.1). The primary advantages over previous technologies (specifically yeast-two hybrid) are that the protein is used in its fully

processed form, the interactions are in the native environment of the protein, and multi-component complexes can be isolated in a single step.

There are three essential components for successfully identifying neuronal proteins using MS-based proteomics: immunoprecipitation of the protein bait, purification of the complex, and the identification of the interacting partners.

The ability to immunoprecipitate a protein bait of interest from the desired system must be established before further isolation of the complex can occur. Ideally, an antibody is available allowing immunoprecipitation of the native protein from tissues. If this is the case, then the bait can be an endogenous protein in native neuronal tissue. However, suitable antibodies are often not readily available. In these instances, the bait protein can be affinity-tagged and expressed in an appropriate cell system, followed by immunoprecipitation with commercially available antibodies directed against the affinity tag.

The coupling of MS technologies with successful co-immunoprecipitation allows rapid and specific identification of discrete members of the protein complex. Importantly, this technique has identified proteins that had previously been shown to interact with the bait of interest using other technologies, reinforcing the efficacy of using MS to identify isolated protein interactors. Finally, several studies using neuronal receptors as bait proteins showed surprisingly little overlap among the interactors that make up the various complexes, speaking to the specificity of the complexes and the detection capabilities of the application. Taken together, these data indicate that MS-coupled co-immunoprecipitation provides a rapid, sensitive, and reliable approach to identifying protein interactors, and that it is an attractive alternative for the discovery of novel interacting partners that cannot be detected using yeast-two hybrid analyses.

This unit provides two methods for performing MS-based proteomics studies to determine the interacting partners of a given target protein (Fig. 5.28.1). The first method focuses on the identification of proteins using transfected cells and epitope-tagged bait proteins (see Basic Protocol 1), while the second focuses on protein identification from native systems with endogenous bait proteins (see Basic Protocol 2). Additionally, while MS-based proteomics can provide a sensitive and effective tool for identification of interacting proteins, this procedure relies on a relatively strong affinity between bait and targets. Furthermore, many biological interactions are transient in nature and depend on cellular environment. These limitations, as well as proper controls, manipulations, and optimization techniques designed to avoid these pitfalls, are discussed in this unit. When used to its full capability, co-immunoprecipitation coupled with MS analysis provides a powerful tool for discovering previously unknown protein partners, thus increasing the understanding of dynamic protein regulation and control.

BASIC PROTOCOL 1

IDENTIFICATION OF PROTEIN-PROTEIN INTERACTIONS USING AN AFFINITY-TAGGED BAIT PROTEIN EXPRESSED IN CULTURED CELLS

This protocol employs cultured cells expressing an affinity-tagged bait protein. It allows expression of a protein of interest for subsequent analysis of other proteins that interact with it in a given cell line. The protocol has two primary strengths. It allows study of a target protein for which no antibody is available for immunoprecipitation, and it allows for very high expression of the bait protein, at levels much higher than may be achieved in an endogenous system. Both of these factors may increase the success of identifying interacting protein partners for a protein of interest. A potential weakness is that it relies on an artificial system so the interactions should be verified for endogenous/physiological importance.

Materials

Plasmid for expressing FLAG-tagged protein of interest

Cultured cells of interest (cell line that originated from the endogenous tissue of interest, e.g., neurons for neuronal bait proteins, or cells capable of high levels of expression)

Cell culture medium

Transfection reagent/method (e.g., lipofectamine from Invitrogen, Neuroporter from Genelantis, or calcium phosphate from BD biosciences)

Earle’s buffered salt solution (EBSS; or similar buffer suited for mammalian cell culture work) containing 5 mM EDTA

Solubilization buffer (see recipe), ice cold

Protease inhibitor cocktail (e.g., Complete Mini from Roche)

Protein-A or -G agarose beads (or other conjugated agarose beads, matched for antibody class and species used in the IP; consult the protein agarose manufacturer for a list of species compatibility; alternatively, a blend of protein A/G agarose may be used)

Agarose beads coupled to anti-FLAG antibody (e.g., Sigma anti-M2 agarose)

Tris-EDTA (TE) buffer, pH 7.4

Protein sample buffer (e.g., 2× LDS sample buffer from Invitrogen)

Reducing agent (e.g., sample reducing agent from Invitrogen)

Pre-cast acrylamide gels (4% to 12% Bis-Tris or similar)

Running and transfer buffer for acrylamide gels

Antibody directed against protein of interest (or against epitope tag)

G-250 Coomassie stain (see recipe) or commercially available colloidal Coomassie stain (e.g., Simply Blue SafeStain from Invitrogen)

Coomassie de-stain (see recipe)

Mass spectroscopy-compatible silver stain (e.g., Silver Quest from Invitrogen), optional

150-mm tissue culture plates

Cell culture incubator

10-ml serological pipets

50-ml conical tubes

Refrigerated centrifuge

Orbital shaker, 4°C

1.5-ml screw-top microcentrifuge tubes with rubber sealing O-rings (e.g., screw-top tubes from Axygen Scientific)

37°C water bath

Gel running and blotting system (e.g., Novex mini-cell and X-Cell II blot module from Invitrogen)

PVDF membranes (e.g., Invitrolon from Invitrogen) or nitrocellulose membranes

Gel photographing equipment

Sterile razor blades

Additional reagents and equipment for PCR (Chapter 4), cell transfection (*APPENDIX 1*)

NOTE: It is critical that the samples be treated as sterile throughout the experimentation. MS analysis is extremely sensitive and any common contaminating proteins (i.e., keratin) can ruin protein detection. Always wear gloves, and use sterile sample tubes and filter-tipped pipets and pipet tips.

Generate expression construct of protein of interest

1. Use PCR to generate an expression plasmid for the fusion protein of interest with an in-frame FLAG-tag (DYKDDDDK) or other easily immunoprecipitated epitope tag (e.g., HA—YPYDVPDYA).

   Many expression constructs for thousands of proteins are available commercially (e.g., Trueclones from Origene). Clones can also be obtained from a collaborator, or self-cloned from libraries.

   A further discussion of epitope tag selection is presented in Critical Parameters. It is also possible to use the native form of the bait protein in conjunction with an antibody that can effectively immunoprecipitate the protein. Such methodology is covered in Basic Protocol 2.

2. Generate a negative control expression plasmid using the same backbone plasmid as the protein of interest, but lacking coding for that protein.

   The plasmid should however express the epitope tag peptide used for step 1.

Express fusion constructs in mammalian cells

3. Seed cells in 150-mm tissue culture plates at a density of 5×10^6 cells (or other appropriate density as required for specific cells) in culture medium and incubate overnight at 37°C. Plate at least one plate for transfection of the protein of interest and one plate for transfection of the negative control plasmid.

4. The next day, aspirate medium and replace with fresh medium, then transfect cells using an appropriate technique (e.g., see *APPENDIX 1* for several methods). Typically, use ~30 μg of DNA per plate. Incubate cells for ~48 hr, changing medium as necessary for the transfection technique.

Harvest transfected cells and solubilize proteins

5. Aspirate medium and add 10 ml warm (37°C) EBSS containing 5 mM EDTA per plate. Return plate to incubator for 10 min.

6. Remove cells by gently washing up and down with a 10-ml serological pipet, and transfer to a 50-ml conical tube. Wash plate with EBSS and add wash to tube containing the cells. Fill tube up to 50 ml with EBSS.

7. Centrifuge cells 10 min at 100 × *g*, 4°C. Discard the supernatant. Lyse the cells by resuspending them (using a 1000-μl pipettor with the tip trimmed to increase the size of the opening) in 1 ml of ice-cold solubilization buffer containing protease inhibitor cocktail. Keep samples on ice or at 4°C at all times.

   Solubilization buffer with protease inhibitors should be prepared and used fresh to avoid degradation of protease inhibitors. If using Complete Mini protease inhibitor cocktail pellets, dissolve one pellet in 50 ml of cold solubilization buffer just prior to resuspending cells. After resuspending cells, leave them in the 50-ml conical tubes.

8. Incubate the resuspended cells/proteins on an orbital shaker 1 hr at 4°C.

9. Remove lysed cells/protein and cellular debris from conical tubes using a 1000-μl pipettor with the pipet tip trimmed to open bore size and transfer to a 1.5-ml screw-top rubber O-ringed microcentrifuge tube (lysate should be clear with clumps of viscous cellular debris). Microcentrifuge 40 min at 20,000 × *g*, 4°C. Retain supernatant and discard the cellular debris pellet.

Pre-clear lysate

10. While centrifuging in step 9, wash the protein-G agarose beads by placing 100 μl of protein-G agarose beads into new 1.5-ml screw-top microcentrifuge tubes (use a trimmed pipet tip or wide-bore pipet tip to avoid shearing the protein G from the agarose beads). Add 1 ml of 4°C solubilization buffer to the tube and gently mix by inverting tube. Microcentrifuge 1 min at ~1500 × *g*, 4°C. Discard supernatant by aspirating with a pipet and repeat.

11. Place supernatant from step 9 into the microcentrifuge tubes containing the washed protein-G agarose beads. Incubate the mixture on a rocking platform for >3 hr at 4°C.

    Failure to perform this pre-clearing greatly increases the amount of non-specific proteins pulled down in the assay.

12. Centrifuge tubes 5 min at 1000–2500 × *g*, 4°C. Retain the supernatant and discard the protein-G bead pellet.

13. Transfer the supernatant into fresh tubes (optionally save 50 µl of the supernatant at this step for analysis as lysate control later) and add 100 µl slurry (50 µl of beads) of agarose beads bound to an antibody directed against the affinity tag on the bait protein (e.g., Sigma anti-M2 agarose). Incubate the mixture on a rocking platform overnight at 4°C.

Wash bead complexes—All wash buffers should be at 4°C, and contain the protease inhibitor cocktail. Centrifugations involving beads should not exceed 2500 × *g*, as the integrity of the beads may be affected.

14. Centrifuge beads 5 min at 1000–2500 × *g*, 4°C. Carefully remove the supernatant and save for later analysis (this can be used to determine the efficiency of the immunoprecipitation reaction by analyzing this supernatant for the presence of the protein of interest via immunoblotting). To ensure removal of all supernatant, centrifuge the tubes again in a benchtop mini-microcentrifuge for 1 min at ~1500 × *g*. Discard supernatant.

15. Resuspend/wash the pelleted beads by pipetting 1 ml ice-cold solubilization buffer and inverting the tubes several times. Centrifuge beads 5 min at 1000–2500 × *g*, 4°C. Discard the wash buffer and repeat for a total of three washes. After the last wash, centrifuge the beads an additional time to ensure that all solubilization buffer is removed.

    Washing is one of the most critical steps to ensure that the number of specific interacting proteins is maximized while limiting the number of nonspecifically interacting proteins. The number, volume, time of wash, and buffer used (i.e., adding a higher salt wash buffer, e.g., IP washing buffer; see recipe) should be determined experimentally. These are guidelines to serve as a starting point, and the parameters should be adjusted according to the individual protein being studied.

16. After removal of all solubilization buffer, wash the beads at least one additional time with 4°C TE buffer, pH 7.4, containing protease inhibitor cocktail by resuspending them and then centrifuging as in step 15. Centrifuge an additional time and remove any additional TE buffer, and discard.

    This wash is essential to remove any residual salts that might interfere with the protein separation via SDS-PAGE.

Elute protein complex and separate via SDS-PAGE

17. Resuspend the beads 1:1 in 50 µl of 2× LDS sample buffer containing reducing agent and gently mix with a large bore pipet tip.

18. Incubate the bead/LDS buffer mixture 1 hr submerged in a 37°C water bath.

The authors have had much better elution success by using this method as opposed to the more common method of SDS-PAGE protein preparation of boiling the samples for 10 min.

It is possible to elute the beads in a non-denaturing elution buffer (commercially available, e.g., Pierce) for experiments using native gels.

19. Remove tubes from the water bath and centrifuge 5 min at 2500 × *g*, 4°C.

Separate protein complex via SDS-PAGE—The quality of the acrylamide gel is especially important for these types of experiments, as it is essential to achieve maximum separation with no contaminants when further processing these samples for mass spectroscopy. For this reason commercially produced pre-cast gradient gels are recommended (e.g., 4% to 12% Bis-Tris gels from Invitrogen along with accompanying gel apparatus, running buffer, and transfer buffer all made with high-quality ultrapure water). When selecting gels, it is important to consider well size and number of wells. It is often recommended that a maximum amount of sample be loaded into a well; therefore, it is not advisable to use a gel with a large number of wells as this will limit the loading capacity of each well. It is also possible to run 2-D gels, and the same considerations apply.

Several commercial mass spectrometry facilities suggest not using Tris-tricine gels for MS analysis experiments, and many have limits on gel thickness (i.e., ≤1.5 mm). It is important to discuss these factors with the MS facility before attempting analysis.

Take special care to avoid any direct contact of the gel with any possible sources of contamination.

20. Prepare two acrylamide gels for each experiment—one gel for staining and mass spectroscopy analysis, and one for verification of proper immunoprecipitation via immunoblot analysis. When loading the gels, load the majority of the protein onto the gel for mass spectroscopy. A starting point would be to load 30 µl of the sample (or the maximum volume as limited by well size) in the MS gel, and 10 µl in the immunoblot analysis gel. Run the negative control on the same gel. Leave one empty lane between samples in the event of overflow from the well (Fig. 5.28.2A).

21. Transfer the immunoblot analysis gel onto PVDF or nitrocellulose. Probe the blot with an antibody directed against the protein of interest or, if none exists, against the epitope tag. Perform immunoblot analysis and visualize and verify proper and selective immunoprecipitation (Fig. 5.28.3).

22. Remove the MS gel and stain with Coomassie blue (Fig. 5.28.2A). If using fresh, sterile lab-made stain, stain for 1 to 2 hr and then de-stain with three

changes of de-stain buffer, 1 hr each. If desired, the gel can be left in de-stain overnight at 4°C in a sealed container.

Coomassie blue-based staining (see Materials) is the most appropriate for MS analysis as it is completely compatible with most MS protocols and has a detection limit that ensures detectable results on a mass spectrometer. As with other aspects of sample preparation, it is always advisable to use commercially available, sterile reagents to minimize the risk of protein contamination.

It is also possible to use other staining protocols like silver staining or fluorescent dye staining; however, proteins may become cross linked to the gel and resistant to proteolytic digestion. To avoid this, an MS-compatible silver stain must be used (available from several manufacturers). Furthermore, the protein concentration in bands detectable with this method may be too low for MS identification. Silver staining does however provide a good control to use for optimizing wash procedures (see step 15).

Prepare samples for mass spectroscopy

23. Photograph the stained gel, and print a hard copy of the picture. Label the molecular weight markers. When proceeding to step 24, note the location of each excised band with a number.

24. To excise the protein bands, place the gel on a light box on top of a clean, clear piece of glass and carefully excise each band using a sterile razor blade. Place each excised band into a sterile microcentrifuge tube (0.3- to 1.5-ml) and label carefully. Be sure to label a hard copy picture of the gel with corresponding band labels so that the approximate molecular weight of each protein can be determined (which will help in its identification via MS). Be sure to also excise the corresponding bands in the negative control lane. Even if there is no corresponding band, cut out the empty gel directly across from each band from the IP lane and analyze it to ensure specificity in protein identification.

The protein bands can be frozen for at least 1 month at −20°C until ready for tryptic digestion and mass spectrometric analysis either at a core facility, one’s own laboratory if MS is available, or through a number of commercial suppliers that supply protein identification services (e.g., www.prottech.com) (Fig. 5.28.2B).

BASIC PROTOCOL 2

IDENTIFICATION OF PROTEIN-PROTEIN INTERACTIONS USING ENDOGENOUS BAIT PROTEINS IN BRAIN TISSUE

This protocol uses endogenous brain tissue that naturally expresses the bait protein of interest. This is a powerful way to identify protein interactions with physiological importance because the interactions are taking place in an endogenous environment. Furthermore, if knockout animals not expressing the bait protein are available, they make an

ideal control because the primary antibody can be used in both test and control reactions. However, a disadvantage to this approach is that an antibody that is specific and capable of precipitating the bait protein must be available. In addition, endogenous tissues generally express much less bait protein than transfected cells, thereby potentially testing the detection limits of the mass spectrometer. If a suitable antibody is available, this protocol is a powerful technique to establish protein interactions in the brain.

Materials

Wild-type mice

Knockout mice lacking protein of interest (optional)

Liquid N_2

Solubilization buffer (see recipe), ice cold

Protein-A or -G agarose beads (or other conjugated agarose beads, matched for antibody class and species used in the IP; consult protein agarose manufacturer for a list of species compatibility; or alternately, use a blend of protein A/G agarose)

Non-immune antibody (monocolonal control) or pre-immune sera (polyclonal control) of same species and type

Antibody to protein of interest with demonstrated ability to immunoprecipitate

Mincing dishes or small beakers

Surgical scissors

50-ml centrifuge tubes

Polytron electric homogenizer with a small probe

1.5-ml screw-top sterile centrifuge tubes with rubber sealing rings (e.g., Axygen Scientific)

Refrigerated microcentrifuge

Rocking platform, 4°C

Additional reagents and equipment for washing beads, eluting and separating protein, and preparing samples for mass spectrometry (see Basic Protocol 1)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Harvest brains and solubilize proteins

1. After sacrificing animals, rapidly remove and freeze brains immediately in liquid N_2. Allow N_2 to dissipate and store for at least 1 year, if flash frozen, at −80°C until ready for assay.
2. Place frozen mice brains in a mincing dish or small beaker and add ~5 ml of ice-cold solubilization buffer. Mince brain with surgical scissors into ~3- to 5-mm size

pieces. Swirl and discard buffer (this serves as a wash to remove any residual blood).

It is possible to change the detergent in the solubilization buffer for purposes of solubilizing more or less protein and altering the stringency protein-protein interactions. The authors have had success replacing 1% Triton X-100 with either 1% digitonin or 1% CHAPS. See Critical Parameters for further discussion of detergent choice.

3. Transfer pieces to a 50-ml centrifuge tube and add 6 ml ice-cold solubilization buffer. Mince pieces with a Polytron electric homogenizer with a small probe attached 15 to 30 sec at 15,000 rpm.
4. Incubate lysate on ice for 1 hr with shaking to solubilize proteins.
5. Remove lysed cells/protein and cellular debris from conical tubes using a 1000-µl pipet with the large-bore pipet tip and equally divide lysate into 1.5-ml screw-top centrifuge tubes with rubber sealing rings (four tubes per brain). Microcentrifuge 40 min at 20,000 × *g*, 4°C.
6. Transfer the supernatant to a new tube and discard the pellet containing cell debris and nonsoluble brain fractions.
7. Centrifuge the lysate 10 min at 20,000 × *g*, 4°C. Transfer the supernatant to a new tube and discard any pellet.

Pre-clear lysate

8. While centrifuging in steps 5 through 7, wash protein-G agarose beads. Place 100 µl protein-G agarose beads into new 1.5-ml screw-top centrifuge tubes. Add 1 ml of 4°C solubilization buffer to tube and mix gently. Microcentrifuge 1 min at ~1500 × *g*, 4°C. Discard supernatant and repeat.
9. Place supernatant from step 7 into the centrifuge tubes containing the washed protein-G agarose beads. Add an appropriate concentration of non-immune IgG or pre-immune sera of the same species and subtype that is going to be used to precipitate the protein of interest. Incubate the mixture on a rocking platform >3 hr at 4°C.

   Failure to perform this pre-clearing typically increases the amount of non-specifically interacting proteins pulled down in the assay; however, one may choose to preclear using only the protein-G beads and not include the non-immune IgG/pre-immune sera. The need for the IgG/sera may have to be determined experimentally based on the amount of non-specifically interacting proteins observed after gel staining.

10. Centrifuge tubes 5 min at 2500 × *g*, 4°C. Discard the protein-G beads pellet.

Immunoprecipitate the complex

11. To the experimental tubes, add ~10 μg of antibody directed against the protein of interest. If using only wild-type animals, add nonspecific IgG or pre-immune sera to the control tubes. If using samples from a knockout animal, add the specific antibody to both wild-type and knockout animal lysates. Incubate on an orbital shaker overnight at 4°C.

12. Place the contents of the tube into a fresh tube containing washed protein-G agarose beads. Incubate the mixture on a rocking platform at 4°C for >3 hr or overnight.

To wash the protein-G agarose beads, place 100 μl of protein-G agarose beads into new 1.5-ml screw-top centrifuge tubes. Add 1 ml of 4°C solubilization buffer to the tube and mix gently. Microcentrifuge 1 min at *~1500 × g, 4°C. Discard supernatant and repeat.*

13. Proceed to Basic Protocol 1, step 14.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Coomassie de-stain

50 ml methanol

50 ml acetic acid

400 ml H_2O

Store up to 1 year at room temperature

G-250 Coomassie stain

1.2 g G-250

300 ml methanol

60 ml acetic acid

Bring up to 500 ml with water

Store up to 1 year at room temperature

IP washing buffer

50 mM HEPES

1 mM EDTA

10% glycerol

1% Triton X-100

500 mM NaCl

50 mM NaF

40 nM $Na_4P_2O_7$, pH 7.4

Store up to 1 year at 4°C

Solubilization buffer

50 mM HEPES

1 mM EDTA

10% glycerol

1% Triton X-100

150 mM NaCl

50 mM NaF

40 mM $Na_4P_2O_7$, pH 7.4

Store up to 1 year at 4°C

COMMENTARY

Background Information

Prior to the complete sequencing of the human genome and advances in peptide sequencing via mass spectroscopy, protein-protein interactions were usually identified via yeast two-hybrid analysis. While this method has identified many important interactions, it suffers from several flaws. Chief among these is the use of only a portion of the target protein for bait, leading to high false-positive findings, as well as missing many interactions that require the entire protein. In addition, yeast two-hybrid analysis is performed outside a native cellular environment, thereby excluding any interaction that is influenced by the physiological environment of the protein.

The convergence of advanced mass spectroscopic techniques and the sequenced genome have allowed for the study of protein-protein interactions within cells. Recently, proteomic-based methods have proven useful for identifying interacting proteins with neurotransmitter receptors, including NMDA (Husi et al., 2000), $P2X_7$ (Kim et al., 2001), and 5-HT_{2C} (Becamel et al., 2002) receptors. Additionally, the authors have been successful using MS approaches to isolate and identify proteins that interact with D_1 and D_2 receptors (Free et al., 2007). These studies highlight the facility of these techniques to glean valuable insights into protein interactions in neuroscience.

Critical Parameters and Troubleshooting

There are several crucial steps in the procedures described in this unit that need to be optimized for a given target protein. They include choice of detergent for solubilizing proteins, washing time and conditions, epitope tag location, and antibody selection.

Choice of detergent—For any given target protein, multiple detergents may have to be tested to determine the one that provides maximum solubility without loss of protein

interactions. Triton X-100 works well for solubilizing membrane-bound receptor proteins without completely compromising the associated interacting proteins. The authors have also had success in solubilizing membrane-bound receptor constituents with CHAPS or digitonin; notably, these detergents have worked well in co-immunoprecipitations from mouse brain. In general, the least harsh detergent that solubilizes the original target protein is likely the best choice to avoid loss of interacting proteins. As mentioned above, it should also be noted that different detergents may be needed for different tissues and the experimenter should be prepared to re-optimize the detergent when moving between expression systems or native tissue sources.

Choice of tag—When performing experiments using epitope-tagged proteins, choice of the tag can be a critical issue. Some epitope tags can be immunoprecipitated more efficiently than others, primarily due to the commercial availability of anti-epitope agarose. Another consideration is the amount of non-specifically interacting proteins that a given tag may precipitate. If a tag pulls down more than a few proteins, it may obscure the specificity of the proteins interacting with the target. For example, using HEK293T cells, the FLAG epitope tag will nonspecifically bind to about ten proteins under certain wash conditions (R.B. Free, L.A. Hazelwood, and D.R. Sibley, unpub. observ.). The authors find this to be an acceptable number that can always be subtracted out of the experiment. Other affinity tags may work just as well, but they should be evaluated prior to experimenting with a protein of interest.

Washing conditions—The single most variable condition for these types of experiments is often washing. Washing conditions—including duration of wash, buffer selection (including salt and pH), volume of wash buffer, and number of washes—must be optimized for every protein complex and tissue type. The general idea is that one should wash with enough vigor to remove most of the non-specifically interacting proteins, while maintaining all of the proteins that interact specifically with the protein of interest. Fewer washes will likely increase the number of proteins identified, but also retain more non-specific interactions. Alternatively, too many washes may cause dissociation of the protein of interest from its interacting complex. In some situations, merely increasing the time of a wash from a few seconds to 5 min, or increasing the wash volume from 1 ml to 10 ml may be adequate to effectively remove any persistent non-specific interactors. However, it may sometimes be necessary to use a buffer with a different salt concentration to achieve optimal results. It is possible that, for certain systems, the ideal conditions for washing remain elusive. For these situations, it may be necessary to cross-link proteins in the native environment before the start of experimentation. Cross-linking will help to retain the protein complexes of interest while allowing more stringent wash conditions to remove proteins that bind non-specifically to the antibody or protein agarose beads. There are, however, separate complications associated with cross-linking, including artifactual complexing of proteins that do not natively associate. For an extensive review of crosslinkers, see Sinz (2003). These wash conditions must be determined experimentally and, as always, it is advised to begin with the least stringent conditions, adjusting as necessary for the particular protein and system.

Antibody selection—When choosing to do these types of experiments with native (untagged) bait protein, antibody selection is of primary importance. This is due to two factors: specificity and epitope location. For obvious reasons, the antibody must be highly specific to ensure that the identified proteins are in fact associated with the target protein. Specificity can be verified by the presence of the target protein in the precipitate via mass spectroscopy, via immunoblot analysis ideally probing with a different antibody for the same protein of interest, or by probing blots containing samples prepared from knockout animals. Epitope location is also potentially important when selecting an antibody. Most antibody epitopes are designed to be in more hydrophilic regions of the protein, the precise locations where most interacting proteins likely bind. It is of concern then that the antibody itself may actually compete with interacting proteins at the epitope site, thereby removing some proteins from the complex. Alternatively, it is also possible that a given antibody may only precipitate a select population of a given protein as its epitope may be blocked by interacting proteins. Either way, one should be aware of these considerations when choosing antibodies and check for efficiency of precipitation by examining the supernatant/lysate for presence of the protein of interest. These problems often make experiments using tagged fusion proteins as opposed to primary antibodies more suitable for this type of study.

Criteria for a positive interaction—After identification of interacting proteins, but before subjecting said proteins to biological studies, one should determine that the interaction is in fact specific for the protein of interest. Several criteria should be established to help accomplish this task. One important method for greatly reducing the number of false positives is to always subject negative controls (i.e., transfected epitope tag or knockout mouse) to the same MS analysis as the target samples. While this adds to the cost of analysis and identification, it is critical for eliminating proteins that interact non-specifically with the antibody, protein beads or other materials. The occurrence of multiple peptides for a putative protein interactor within the target sample and none within the non-specific control sample also greatly reduces the possibility that the candidate is either interacting non-specifically or is a false positive. More peptides indicate that the interactor is an abundant component of the given sample and therefore likely a major interacting protein within the complex of interest.

Peptide length can also affect results. Longer peptides are less likely to result in incorrect sequence alignments when mining databases for potential protein matches.

Other suggestions include identifying the protein in more than one system, such as in cells and brain, or in multiple cell lines. Identification in more than one system indicates a conserved interaction that likely is important for the bait protein. Identification of the protein in multiple MS samples from the same source is also valuable, as immunoprecipitation followed by MS from each protein source should be repeated at least twice.

Interacting proteins should be identified in at least two independent experiments to be considered significantly valid. If there is suitable evidence from MS analysis to further pursue the candidate interacting protein, verification by biochemical means is necessary. If an antibody to the interacting partner is available, the bait should be immunoprecipitated as described in this unit and an immunoblot probed with an antibody against the interactor. Similarly, the reverse co-immunoprecipitation should also be performed: perform the

immunoprecipitation with an antibody against the novel interactor, and probe the immunoblot with an antibody against the original bait protein of interest.

Finally, for a protein to be considered physiologically relevant, co-localization studies in rodent brain could serve as a final filtering criterion. Following a defined set of criteria should eliminate further study of artifact or non-specifically interacting proteins and help ensure success when examining the biological consequence of novel interactions.

Anticipated Results

After optimization of all critical parameters, it can be anticipated that most proteins will precipitate with some interacting proteins. How many proteins interact with the bait protein is of course dependent on the bait protein. A user's results will be highly specific for a particular protein and system. It can be anticipated that at least some proteins will participate with the bait protein and not the negative control (Fig. 5.28.2). If these proteins are visible via Coomassie staining, it is reasonable to assume that there is a sufficient quantity of protein to be identified via mass spectroscopy (Fig. 5.28.2B). All interacting proteins should be verified by another means such as immunoblot (Fig. 5.28.3) to ensure that the interactions are specific.

Time Considerations

Using the methods described in this unit, it takes several days to prepare samples for mass spectroscopy. Cell culture and transfection require at least 48 hr. The actual immunoprecipitation reaction can be easily broken into 2 days. On the first day, the transfected cells can be harvested and the protein solubilized. Also on the first day, the lysate can be pre-cleared and the overnight immunoprecipitation incubation begun—this all takes ~5 hr. The second day entails washing the beads, eluting the proteins, running the gels, transferring a gel, staining a gel, imaging a gel, and excising bands for mass spectroscopy. This takes ~8 hr. To obtain useful results will however take longer since the wash technique has to be optimized for a given bait protein and cell system. Because this technique is so dependent on optimization, the amount of time required will vary greatly.

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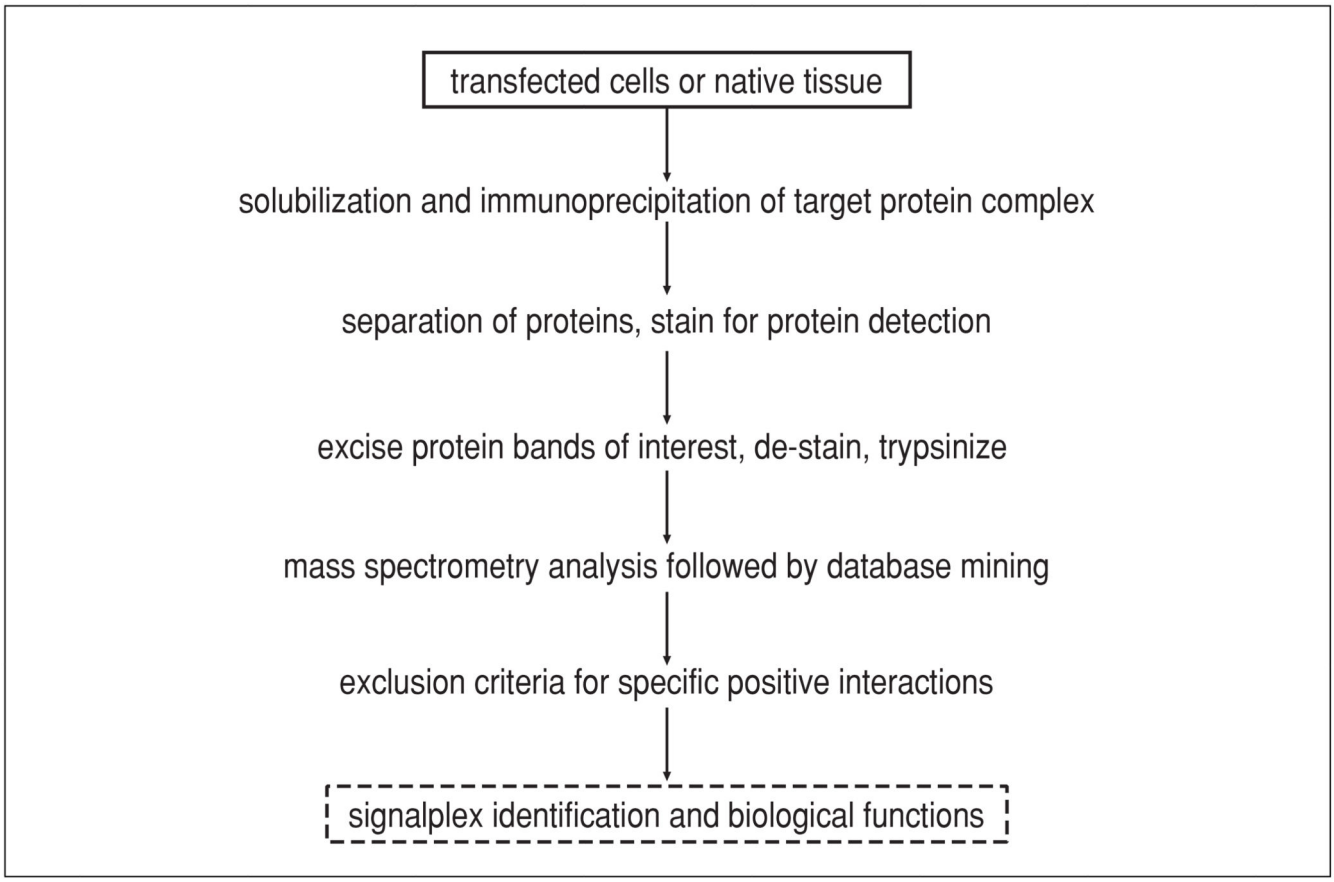

Figure 5.28.1.
Overview of procedure for identifying novel protein-protein interactions using co-immunoprecipitation and mass spectroscopy.

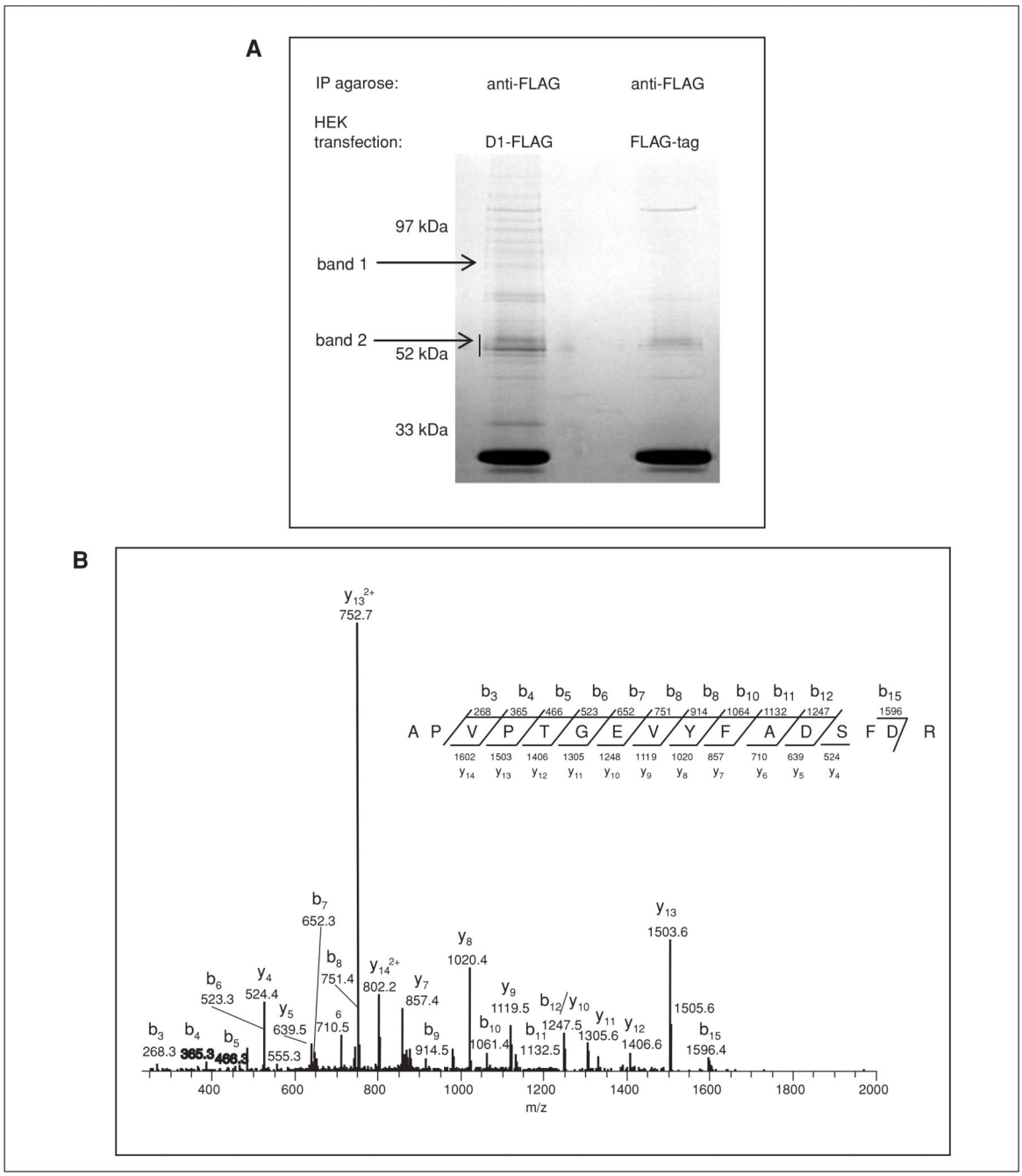

Figure 5.28.2.

Identification of D_1 receptor–interacting proteins using immunoprecipitation and mass spectroscopy. (**A**) Coomassie-blue stained gel of immunoprecipitated proteins. The D_1-FLAG lane shows proteins immunoprecipitated from cells expressing the FLAG-D_1 receptor. The FLAG-tag lane shows proteins immunoprecipitated from cells only expressing the FLAG peptide. Band number indicates the band cut from the gel and subjected to MS-based sequencing. Band 1 was found to be the interacting protein calnexin. Band 2 was found to be the parent bait protein, the D_1 dopamine receptor. Neither of these proteins was

found in the control lane. (**B**) Representative MS/MS spectrum obtained after excision of band 1 and fragmentation of the precursor ion at m/z 886.6. LC-MS/MS (tandem mass spectrometry) was carried out on the peptide mixture obtained from in-gel digestion of SDS-PAGE-separated protein samples with LCQ ion trap mass spectrometer on-line coupled with an HPLC with 75-μm i.d. C18 column. The precursor ions were selected automatically by the instrument. The peptide sequence was identified by bioinformatics analysis and found to correspond to the parent protein calnexin. This figure is adapted from Free et al. (2007), with permission.

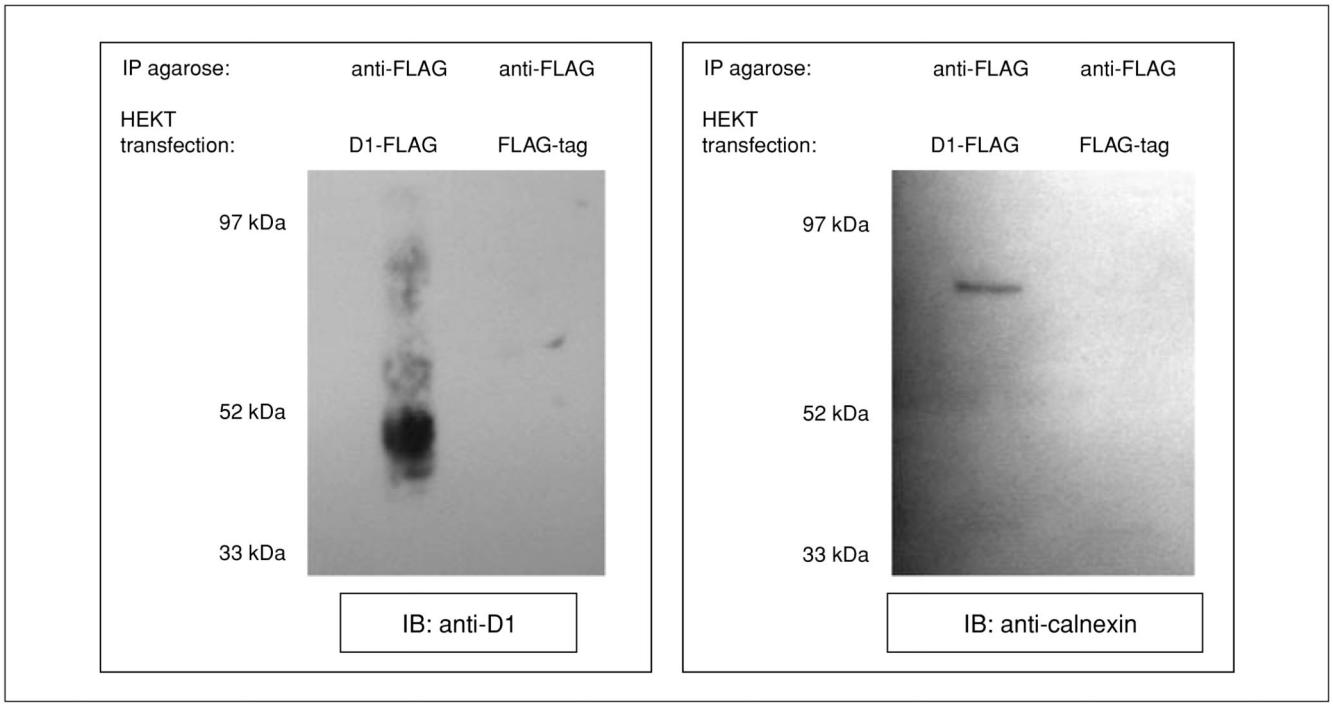

Figure 5.28.3.

Verification of interaction via immunoblot analysis. Left panel: HEK293T cells were transfected with D_1-FLAG or vector containing only the FLAG peptide (FLAG-tag). Proteins were extracted, immunoprecipitated (IP) using anti-FLAG agarose, separated by SDS-PAGE and immunoblotted (IB). Blots were probed with a monoclonal anti-D_1 antibody and visualized using enhanced chemiluminescence (ECL) after incubation with an anti-rat HRP-conjugated antibody. Right panel: The blot in the left panel was stripped of all antibodies, re-probed with an anti-calnexin antibody and visualized using ECL after incubation with an anti-rabbit HRP-conjugated antibody. These data confirm the mass spectroscopy findings in Figure 5.28.2 and support an interaction between the proteins. This figure is adapted from Free et al. (2007), with permission.