CHAPTER 9

CO-IMMUNOPRECIPITATION AS A STRATEGY TO EVALUATE RECEPTOR–RECEPTOR OR RECEPTOR–PROTEIN INTERACTIONS

RANDY A. HALL

9.1 INTRODUCTION

Immunoprecipitation is a useful method for isolating proteins of interest from cellular extracts using specific antibodies. Following immunoprecipitation of a protein of interest, it can be determined via Western blot whether any other proteins have co-immunoprecipitated. This method has been routinely used over the past several decades to study protein–protein interactions and thereby elucidate cellular signaling pathways.

The interactions of G protein-coupled receptors (GPCRs) with a variety of protein partners have proven tractable to analysis via co-immunoprecipitation. For example, it is in some cases possible to co-immunoprecipitate G proteins with GPCRs (Matesic et al., 1989; Law et al., 1991; Matesic et al., 1991; Law and Reisine, 1992; Okuma and Reisine, 1992; Georgoussi et al., 1995; Sidhu et al., 1998; Chalecka-Franaszek et al., 2000). This has been a useful method for helping to characterize the specificity of G protein coupling for certain receptors. The associations of arrestins with GPCRs have also been effectively studied via co-immunoprecipitation (Luttrell et al., 1999; Cheng et al., 2000; Cen et al., 2001; Chen et al., 2002; Conlan et al., 2002; Kishi et al., 2002; Perry et al., 2002), as have interactions between GPCRs and various other cytoplasmic proteins (Table 9.1). Finally, co-immunoprecipitation has been an effective method for studying GPCR dimerization (Table 9.2). Receptor–receptor interactions characterized via co-immunoprecipitation

G Protein-Coupled Receptor–Protein Interactions. Edited by Susan R. George and Brian F. Dowd
ISBN 0-471-23546-6

Table 9.1 List of GPCR Interactions with Cytoplasmic Proteins, Other Than G Proteins and Arrestins, That Have Been Characterized Using Co-immunoprecipitation[a]

Receptor	Interacting Protein	Reference
Adrenergic, α_2	Spinophilin	Richman et al., 2001
Adrenergic, β_1	PSD-95	Hu et al., 2000
Adrenergic, β_1	MAGI–2	Xu et al., 2001
Adrenergic, β_2	EIF–2B	Klein et al., 1997
Adrenergic, β_2	AKAP79	Fraser et al., 2000
Adrenergic, β_2	AKAP250	Shih et al., 1999
Angiotensin, AT_1	Jak2	Ali et al., 1997
Angiotensin, AT_1	ATRAP	Daviet et al., 1999
Bradykinin, B2	eNOS	Ju et al., 1998
Bradykinin, B2	SHP-2	Duchene et al., 2002
Calcium sensing	α-Filamin	Awata et al., 2001
Cannabinoid, CB1	FAN	Sanchez et al., 2001
Complement, C5a	WASP	Tardif et al., 2003
Dopamine, D2/3	α-Filamin	Lin et al., 2001a
GABA, $GABA_BR1$	14–3–3	Couve et al., 2001
Glutamate, mGluR1/5	Homer	Xiao et al., 1998
Glutamate, mGluR1/2/3/5	Tamalin	Kitano et al., 2002
Glutamate, mGluR7	PICK1	Dev et al., 2000
Prolactin-releasing peptide	PICK1	Lin et al., 2001b
Rhodopsin, *Drosophila*	InaD	Xu et al., 1998
Serotonin, 5-HT_2A	PSD-95	Xia et al., 2003
Serotonin, 5-HT_2C	MUPP1	Becamel et al., 2001
Somatostatin, SSTR2	CortBP1	Zitzer et al., 1999

[a]This list is by no means comprehensive, as there are many other examples of GPCR/protein associations that have been shown via co-immunoprecipitation and other techniques. However, a survey of the methods sections of the references listed here may allow for useful comparisons of the varying conditions utilized for co-immunoprecipitation studies by different laboratories. Optimal conditions for solubilization and immunoprecipitation can vary significantly from receptor to receptor.

have been validated using other techniques, including BRET (Angers et al., 2002), fluorescence resonance energy transfer (FRET) (Ramsay and Milligan, this volume, Chapter 3), and physiological studies (Wong et al., this volume, Chapter 15).

There are a number of key technical points to be considered in performing co-immunoprecipitation experiments. First, the choice of the antibodies to be used is of critical importance. Second, the method of receptor solubilization can often have a major impact on the outcome of the studies. Third, the protocol used, especially the stringency of the washes, should be optimized in order to obtain clear and consistent results. Finally, protein modifications such as chemical crosslinking may be utilized to help protein complexes survive the lengthy washes involved in most co-immunoprecipitation protocols. All of these points will be addressed in the sections below.

9.2 ANTIBODY SELECTION

Choosing the right antibodies is a critical first step in any immunoprecipitation study. It is best to have two antibodies that recognize the target protein: one antibody with which to

Table 9.2 List of Examples of GPCR Homo- and Heterodimerization That Have Been Characterized Using Co-immunoprecipitation[a]

Receptor 1	Receptor 2	Reference
Acetylcholine, M3	Acetylcholine, M3	Zeng and Wess, 1999
Adenosine, A_1	Dopamine, D1	Gines et al., 2000
Adenosine, A_1	Purinergic, $P2Y_1$	Yoshioka et al., 2001
Adenosine, A_2	Dopamine, D2	Hillion et al., 2002
Adrenergic, α_{2A}	Adrenergic, β_1	Xu et al., 2003
Adrenergic, β_1	Adrenergic, β_1	He et al., 2002
Adrenergic, β_1	Adrenergic, β_2	Lavoie et al., 2002
Adrenergic, β_2	Adrenergic, β_2	Hebert et al., 1996
Adrenergic, β_2	Opioid, δ	(Jordan et al., 2001)
Angiotensin, AT_1	Bradykinin, B_2	AbdAlla et al., 2000
Calcium sensing	Calcium sensing	Bai et al., 1998
Chemokine, CCR5	Opioid, μ	Suzuki et al., 2002
Cholecystokinin, type A	Cholecystokinin, type A	Cheng and Miller, 2001
Dopamine, D3	Dopamine, D3	Elmhurst et al., 2000
$GABA_BR1$	$GABA_BR2$	Kaupmann et al., 1998
Glutamate, mGluR5	Glutamate, mGluR5	Romano et al., 1996
Opioid, δ	Opioid, δ	Cvejic and Devi, 1997
Opioid, δ	Opioid, κ	Jordan and Devi, 1999
Opioid, δ	Opioid, μ	George et al., 2000
Opioid, μ	Somatostatin, SST2	Pfeiffer et al., 2002
Serotonin, $5\text{-}HT_{1A}$	Serotonin, $5\text{-}HT_1B$	Salim et al., 2002
Somatostatin, SST2	Somatostatin, SST3a	Pfeiffer et al., 2001
Thyrotropin	Thyrotropin	Latif et al., 2001

[a]For the examples of homodimerization shown here, the same receptor is listed under both Receptor 1 and Receptor 2. This list is by no means comprehensive, as there are many other examples of GPCR/GPCR associations that have been shown via co-immunoprecipitation and other techniques. However, a survey of the methods sections of the references listed here may allow for useful comparisons of the varying conditions utilized for receptor/receptor co-immunoprecipitation studies by different laboratories.

immunoprecipitate and another with which to visualize the protein on Western blots. It is possible, of course, to use a single antibody for both the immunoprecipitation and blots, but this is less than ideal for two main reasons. First, the antibody might nonspecifically immunoprecipitate a protein other than the protein of interest. If the same antibody is then used to verify the immunoprecipitation on a Western blot, it may appear to confirm a successful immunoprecipitation, even though the wrong protein was pulled down. Second, if a single antibody is used for both immunoprecipitation and Western blotting, the immunoprecipitated sample will be contaminated with the heavy and light chains of the primary antibody from the immunoprecipitation. These subunits will be strongly recognized by the secondary antibody used in the Western blot, resulting in the formation of intense nonspecific bands that may obscure specific bands of interest. In contrast, if a completely independent antibody is used in the Western blot, and if this antibody is from a different species than the antibody used in the immunoprecipitation, then it is often possible to avoid the problem of nonspecific immunoreactivity in Western blots of the immunoprecipitated sample.

It is important to remember that not all antibodies that work on Western blots will also work for immunoprecipitation. On Western blots, proteins are typically denatured, and

most potential antibody epitopes are therefore fully exposed. However, many of these epitopes may not be accessible to antibodies when the protein is in its native conformation, as in an immunoprecipitation experiment. Thus, the only way to know if an antibody is effective for immunoprecipitation is to experimentally test it. This is yet another reason why it is best to have at least two good antibodies to recognize the protein of interest because the experiment can then be configured in such a way that the antibody that better recognizes the protein in its native state can be used for the immunoprecipitation, while the antibody that better recognizes the denatured form of the protein can be used for the Western blot.

For many GPCRs, high-affinity antibodies that recognize both the native and denatured forms of the receptor are not readily available. For this reason, epitope tagging has been commonly used in immunoprecipitation experiments involving GPCRs. This method involves engineering a short sequence into the receptor expression construct such that the expressed receptor has a small epitope tag that is robustly recognized by antibodies. There is an excellent chapter on this subject in a previous volume of this series (Klein and von Zastrow, 2000). Commonly used epitope tags include Flag, hemagglutinin, myc, and hexahistidine. All of these tags are between 6 and 10 amino acids in length, and a number of high-affinity antibodies that recognize them are commercially available from various suppliers. One additional advantage to working with epitope-tagged receptors is that the antibodies recognizing these tags are often available in a form that is already covalently linked to agarose beads, which can help to make immunoprecipitation experiments easier and more efficient.

One final consideration in antibody selection is that, in experiments aimed at analyzing the association of a GPCR with an intracellular protein, it is preferable to utilize an antibody that recognizes an extracellular region of the receptor. The reason for this is that an antibody directed against intracellular regions of the receptor might potentially compete for binding with associated proteins and thereby disrupt the very interactions that are being examined. For co-immunoprecipitation experiments characterizing GPCR interactions with cytoplasmic proteins, many investigators utilize antibodies directed against either the receptor's extracellular N-terminus or an N-terminal epitope tag, as illustrated in the schematic diagram shown in Figure 9.1.

9.3 RECEPTOR SOLUBILIZATION

For co-immunoprecipitation experiments involving transmembrane proteins such as GPCRs, the choice of solubilization conditions is extremely important. Obviously, the detergent used must be strong enough to extract the receptors from their native membranes. However, the detergent should not be so strong that it denatures the receptors and disrupts protein–protein interactions that might be of physiological interest. There are many detergents available for the solubilization of membrane proteins (le Maire et al., 2000), of which the most commonly used for solubilization of GPCRs are nonionic detergents such as Triton X-100, Nonidet P-40, and CHAPS. However, the ideal solubilization conditions are different for every receptor and should be determined empirically in each case. Moreover, if the retention of receptor activity is important, it should be appreciated that many receptors will become completely inactive (i.e., unable to bind ligands) when solubilized in certain detergents. A classic example is the β_2-adrenergic receptor, which loses its ligand-binding activity when solubilized with most detergents,

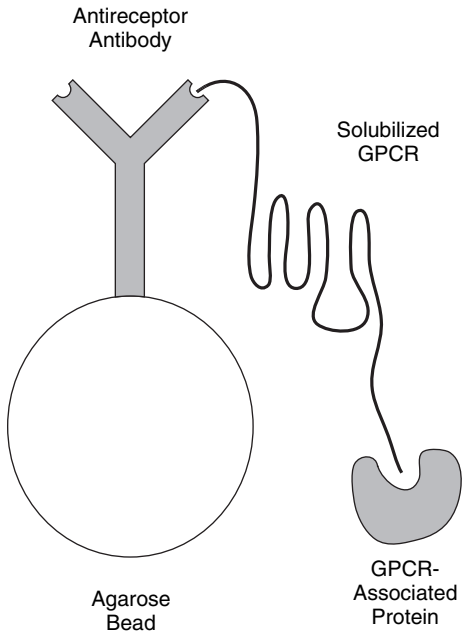

Figure 9.1 Schematic illustration of a co-immunoprecipitation experiment involving a GPCR and an associated protein. An antibody that recognizes either an epitope tag on the receptor or the native receptor itself is linked to an agarose bead. The antibody/bead linkage may be either a direct covalent attachment or via coating of the bead with protein A/protein G. Once the receptor is coupled to the antibody/bead complex, it can be easily precipitated out of solution, allowing for analysis of any proteins associated with the receptor via SDS–PAGE and Western blotting.

yet retains its normal pharmacological profile when solubilized with digitonin (Caron and Lefkowitz, 1976).

The factors that determine the efficiency of receptor solubilization include the detergent chosen, the concentration of the detergent, the ionic strength of the buffer, the length of the solubilization procedure, and the temperature at which it is performed. In general, solubilization will be most efficient with higher detergent concentration, higher ionic strength, longer incubation times, and higher temperatures. However, any potential increase in solubilization efficiency from changing these factors must be weighed against potential losses of receptor activity and potential disruption of protein–protein interactions of interest. A sample protocol for solubilization of a GPCR from either a sample of tissue or cultured cells is provided below.

1. Homogenize the sample thoroughly in a buffer containing 20 mM HEPES [*N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid], pH 7.4, 100 mM NaCl, 1 mM EDTA (ethylene diaminetetraacetic acid), and protease inhibitors. The buffer should be kept ice-cold to minimize proteolysis. Ideally, the protein concentration of the sample should be in the range of 1 to 2 mg protein per mL.
2. Add detergent to the sample to achieve a final concentration of 1.0% and then incubate the sample at 4°C with end-over-end agitation for 1 h.

3. Centrifuge the sample (15,000*g*) for 20 min at 4°C to remove the membrane and any unsolubilized proteins. If the sample does not look completely clear after centrifugation, pour off the supernatant into a fresh tube and centrifuge it again. It is very important that the sample contain no insoluble material, since any insoluble aggregates will tend to stick to the beads during the immunoprecipitation steps and show up as nonspecific background bands on the Western blots at the end of the experiment.
4. Following centrifugation, incubate the solubilizate with agarose beads (yielding a bead bed of 30 μL/mL sample) for 30 min at 4°C with end-over-end agitation, then centrifuge the sample for 30 s at 3000*g*. This "preclearing" step will help to remove proteins in the sample that are prone to nonspecifically attaching to the beads.
5. Remove the supernatant, leaving behind the beads, and place the supernatant in a fresh tube. The sample is now ready for immunoprecipitation.

9.4 PROTOCOL FOR IMMUNOPRECIPITATION

As mentioned above, immunoprecipitation protocols should be optimized for each receptor. That being said, here is a standard protocol for immunoprecipitation of a GPCR followed by an examination via Western blot for co-immunoprecipitated proteins. The volumes given will assume that the solubilized sample is 1.0 mL. For samples of different starting volumes, the protocol can be scaled accordingly.

1. Remove a small fraction (50 μL) of the solubilizate from the final step of the solubilization protocol described above, and incubate this small aliquot of solubilizate with SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) sample buffer in order to examine the expression of proteins in the starting lysate fraction.
2. To the remaining solubilizate (950 μL), add 30 μL of Protein A/G agarose beads (Sigma) plus 2 μg of antibody directed against the GPCR of interest. Alternatively, if the GPCR of interest is epitope-tagged, add 30 μL of agarose beads that are prelinked to the appropriate antibody (i.e., anti-Flag affinity agarose from Sigma). Incubate the solubilizate/bead mixture for 2 h at 4°C with end-over-end rotation.
3. Centrifuge the sample for 30 s at 3000*g* and remove the supernatant. Add 1.0 mL of fresh, ice-cold solubilization buffer to the beads, and vortex the sample vigorously.
4. Repeat step "3 two to five more times, such that the beads are washed 3three to six times total. Washing more times here will increase the stringency of the co-immunoprecipitation, while washing fewer times will decrease stringency.
5. After the final wash, add 100 μL of 1× SDS–PAGE sample buffer to the beads in order to denature and elute the immunoprecipitated proteins. Vortex the tube and incubate for 30 min in a 37°C water bath to facilitate mixing of the sample. Some investigators boil the sample for 5 min during this step, which can help to speed the elution process but can also in some cases result in enhanced protein aggregation on SDS–PAGE gels.
6. Load the samples into lanes of SDS–PAGE gels and run the gels for 1 h at 150 V, then transfer to nitrocellulose by incubating the gel and nitrocellulose together for

1.5 h at 25 V (times and voltages may vary depending on what type of apparatus is used).

7. Block the resultant nitrocellulose blots with "blot buffer" (2% nonfat dry milk, 0.1% Tween-20, 50 mM NaCl, 10 mM Hepes, pH 7.4) for at least 30 min, then incubate with the primary antibody in blot buffer for 1 h at room temperature.
8. Wash the blot three times for 5 min with 10 mL of blot buffer and then incubate for 1 h at room temperature with an appropriate horseradish-peroxidase-conjugated secondary antibody in blot buffer.
9. Again, wash the blot three time for 5 min with 10 mL blot buffer, rinse briefly with phosphate-buffered saline, and then visualize the proteins of interest via enzyme-linked chemiluminescence (ECL kit from Amersham Pharmacia Biotech).

9.5 IMPORTANT CONTROLS

There are several controls that should be performed in order to verify that immunoprecipitation has occurred. First, the immunoprecipitated sample(s) should be run on the same SDS–PAGE gel alongside a sample of the starting lysate and one or more mock-immunoprecipitated samples. An antibody directed against the immunoprecipitated protein (referred to here as protein X) should recognize a band of exactly the same size in both the lysate and the immunoprecipitated samples, but not in the mock-immunoprecipitated samples. There are two general ways to do a mock immunoprecipitation: (i) perform the entire immunoprecipitation process with beads only, either including no antibody or an irrelevant antibody, or (ii) if a peptide to block the immunoprecipitating antibody is available, perform the standard immunoprecipitation protocol except preincubate the antibody/bead mixture with a saturating dose of the blocking peptide before adding it to the lysate in order to prevent specific binding of the antibody to protein X. The latter method of mock immunoprecipitation is preferred, but the former may be used if there is not a blocking peptide available.

To confirm the specificity of any observed co-immunoprecipitation, controls similar to those described above should also be performed. That is to say, a band corresponding to the putative co-immunoprecipitating protein (referred to here as protein Y) should be present in the starting lysate and in the immunoprecipitated sample(s) but not in the mock immunoprecipitated sample(s). Moreover, if the experiments are being performed in transfected cells, it is most convincing if side-by-side immunoprecipitation experiments are performed on cells singly transfected with protein Y in addition to cells doubly transfected with both proteins X and Y. If immunoprecipitation of protein Y is observed in the presence but not in the absence of co-transfection with protein X, this represents strong evidence for specific co-immunoprecipitation of the two proteins (Fig. 9.2).

In studying interactions between overexpressed GPCRs using co-immunoprecipitation, there has been some concern that the receptors' hydrophobic domains might nonspecifically aggregate together after solubilization, yielding artifactual co-immunoprecipitation results (Angers et al., 2002). One way this concern has been addressed has been via additional controls where the two receptors of interest are transfected separately into different sets of cells. The cells are then harvested and solubilized, and the solubilizates are mixed together to allow the solubilized receptors time to mingle prior to immunoprecipitation. Typically, these experiments have revealed no evidence for co-immunoprecip-

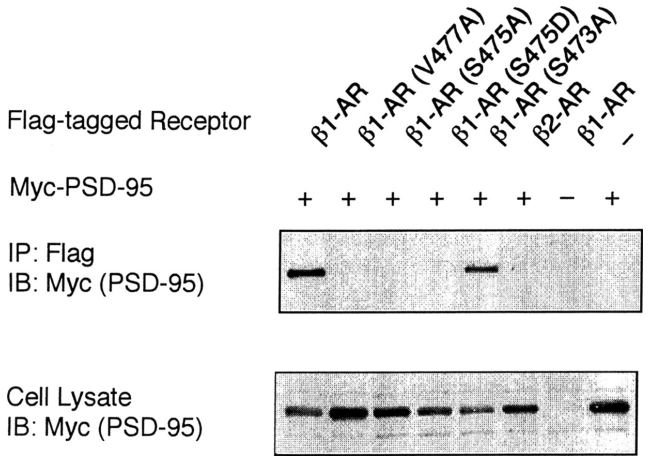

Figure 9.2 Example of co-immunoprecipitation of a GPCR and associated cytoplasmic protein. In this case, the experiment was designed to test the interaction of the β_1-adrenergic receptor (β_1AR) with the scaffold protein PSD-95. HEK-293 cells were transiently transfected with pcDNA3/Flag-β_1AR, pcDNA3/Flag-β_2AR, and/or GW1/Myc-PSD-95. Forty-eight hours posttransfection, the Flag-tagged receptors were solubilized with a buffer containing 1% Triton X-100 and immunoprecipitated using anti-Flag affinity agarose. Myc-PSD-95 bound to the beads was resolved on SDS–PAGE and Western blotted with an anti-Myc antibody (upper panel). Co-immunoprecipitation of PSD-95 was detected with wild-type β_1AR but not with the closely related β_2AR, nor with several β_1AR mutants (V477A, S475A, S475D) harboring single amino acid substitutions at the receptor's distal carboxyl-terminus. The lack of co-immunoprecipitation observed with β_2AR and the β_1AR point mutants strengthens the case that the co-immunoprecipitation observed with the wild-type β_1AR is specific. The expression levels of PSD-95 in the cell lysates are shown in the bottom panel.

itation of receptors that are expressed separately and then mixed together (see many of the references listed in Table 9.2), suggesting that the receptors must be expressed in the same cells in order to associate.

9.6 PROTEIN CROSSLINKING

Immunoprecipitation protocols typically require multiple washes, which take a number of minutes to complete, in order to separate specifically bound proteins from proteins that are nonspecifically associated with the beads. Many physiologically relevant protein–protein interactions have off-rates on the order of seconds, not minutes, and such interactions can therefore be difficult to detect via co-immunoprecipitation. To combat this problem, some investigators have successfully utilized covalent protein–protein crosslinking to help stabilize interactions of interest between GPCRs and various associated proteins. For example, crosslinking is often necessary for the successful co-immunoprecipitation of GPCR/arrestin complexes (Luttrell et al., 1999; Cheng et al., 2000; Cen et al., 2001; Kishi et al., 2002; Perry et al., 2002), although the interactions of some GPCRs with arrestins can be robustly detected via co-immunoprecipitation in the absence of crosslinking (Chen et al., 2002; Conlan et al., 2002).

There are a number of protein crosslinking reagents that are commercially available. Most of them are bifunctional molecules that react with either amine groups or thiol groups. The crosslinker that probably has been most commonly used in studies with GPCRs is DSP (dithiobis[succinimidylpropionate]), available from Pierce, which is a bifunctional amine-reactive crosslinker that is cell permeable and cleavable by reducing agents. The cleavability is an important feature, since DSP will stabilize protein–protein interactions in a cellular context but will not continue to tether the proteins together under the reducing conditions of SDS–PAGE. Thus, proteins will run at their normal size and not as a complex. In a standard protocol, cells are incubated in media lacking serum and containing 1 mM DSP for an hour at room temperature. The cells are then washed to remove excess crosslinker and harvested for solubilization. A more extensive chapter on protein crosslinking involving GPCRs may be found in this volume (Javitch et al., Chapter 6).

9.7 OPTIMIZING THE PROTOCOL

As with many techniques for studying protein–protein interactions, there are two general types of problems with co-immunoprecipitation experiments: false positives and false negatives. False positives may occur if the washing steps are not stringent enough, while conversely false negatives may occur if the washing is too stringent. The stringency of washing may be modified by adjusting several different factors:

1. *Number of washes:* More washes will lead to increased stringency; less washes will lead to decreased stringency.
2. *Volume of washes:* Larger wash volumes are more stringent; smaller wash volumes are less stringent.
3. *Detergent concentration:* Higher detergent concentration will lead to greater stringency; lower detergent concentration will lead to lesser stringency.
4. *Ionic strength of buffer:* Higher ionic strength buffer contributes to more stringent washing; lower ionic strength buffer contributes to less stringent washing. Thus, increasing the salt concentration of the washing buffer will increase washing stringency, but using salt concentrations over 300 mM NaCl is not recommended since even physiologically relevant protein–protein interactions can be disrupted at salt concentrations above this range.

9.8 CONCLUDING THOUGHTS

Co-immunoprecipitation is an extremely useful method for studying both GPCR dimerization (Fig. 9.3) and interactions between GPCRs and other proteins. This technique can be utilized in the study of both native tissues and transfected cells. However, it is important to note that while co-immunoprecipitation can demonstrate that two proteins are found in the same cellular complex, it can never prove that the two proteins are actually physically touching one another. Since co-immunoprecipitation experiments are performed using cell lysates, it is always possible that two co-immunoprecipitating proteins may be linked together by a third protein that acts as a scaffold. For this reason, it is ideal to combine co-im-

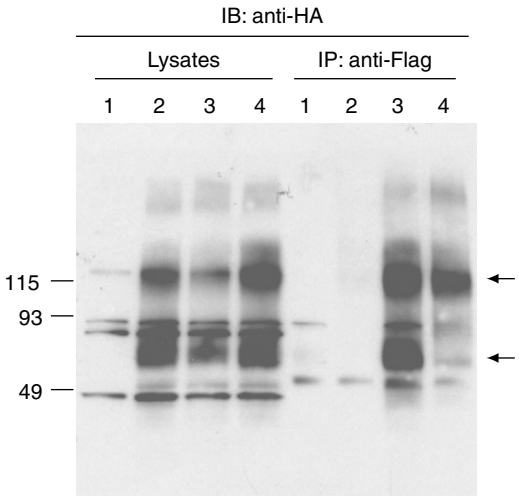

Figure 9.3 Example of co-immunoprecipitation of GPCR heterodimers. In this case, the experiment was designed to examine potential heterodimerization between α_{2A}-adrenergic receptors (α_{2A}AR) and β-adrenergic receptors (βAR). HEK-293 cells were transfected with (1) empty vector, (2) HA-α_{2A}AR alone, (3) HA-α_{2A}AR/Flag-β_1AR, or (4) HA-α_{2A}AR/Flag-β_2AR. The expression levels of HA-α_{2A}AR in Triton X-100-solubilized lysates prepared from the transfected cells are shown in the first four lanes of this Western blot. Several nonspecific bands were evident in untransfected cell lysates (lane 1), whereas specific immunoreactivity for HA-α_{2A}AR (lanes 2 to 4) was observed as major bands at approximately 65 and 120 kDa (arrows). Like immunoreactivity for many GPCRs, α_2AR immunoreactivity on Western blots is often observed as multiple bands, probably corresponding to monomers and SDS-resistant dimers. The solubilized lysates in this experiment were incubated with anti-Flag affinity agarose to immunoprecipitate the Flag-tagged β-adrenergic receptors, and the resultant immunoprecipitates were examined via Western blot for anti-HA immunoreactivity. As shown in the last two lanes of this figure, specific co-immunoprecipitation of HA-α_{2A}AR was observed with both Flag-β_1AR and Flag-β_2AR. The positions of molecular mass standards are indicated on the left side of the figure.

munoprecipitation experiments whenever possible with techniques that utilize purified proteins, such as fusion protein pull-downs (Lee and Liu, this volume, Chapter 12), in order to help assess whether a given GPCR/protein interaction is direct or indirect.

REFERENCES

AbdAlla S, Lother H, Quitterer U (2000): AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. Nature 407:94–98.

Ali MS, Sayeski PP, Dirksen LB, Hayzer DJ, Marrero MB, Bernstein KE (1997): Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT1 receptor. J Biol Chem 272:23382–23388.

Angers S, Salahpour A, Bouvier M (2002): Dimerization: An emerging concept for G protein-coupled receptor ontogeny and function. Annu Rev Pharmacol Toxicol 42:409–435.

Awata H, Huang C, Handlogten ME, Miller RT (2001): Interaction of the calcium-sensing receptor and filamin, a potential scaffolding protein. J Biol Chem 276:34871–34879.

Bai M, Trivedi S, Brown EM (1998): Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. J Biol Chem 273:23605–23610.

Becamel C, Figge A, Poliak S, Dumuis A, Peles E, Bockaert J, Lubbert H, Ullmer C (2001): Interaction of serotonin 5-hydroxytryptamine type 2C receptors with PDZ10 of the multi-PDZ domain protein MUPP1. J Biol Chem 276:12974–12982.

Caron MG, Lefkowitz RJ (1976): Solubilization and characterization of the beta-adrenergic receptor binding sites of frog erythrocytes. J Biol Chem 251:2374–2384.

Cen B, Yu Q, Guo J, Wu Y, Ling K, Cheng Z, Ma L, Pei G (2001): Direct binding of beta-arrestins to two distinct intracellular domains of the delta opioid receptor. J Neurochem 76:1887–1894.

Chalecka-Franaszek E, Weems HB, Crowder AT, Cox BM, Cote TE (2000): Immunoprecipitation of high-affinity, guanine nucleotide-sensitive, solubilized mu-opioid receptors from rat brain: Coimmunoprecipitation of the G proteins G(alpha o), G(alpha i1), and G(alpha i3). J Neurochem 74:1068–1078.

Chen Z, Dupre DJ, Le Gouill C, Rola-Pleszczynski M, Stankova J (2002): Agonist-induced internalization of the platelet-activating factor receptor is dependent on arrestins but independent of G-protein activation. Role of the C terminus and the (D/N)PXXY motif. J Biol Chem 277:7356–7362.

Cheng ZJ, Miller LJ (2001): Agonist-dependent dissociation of oligomeric complexes of G protein-coupled cholecystokinin receptors demonstrated in living cells using bioluminescence resonance energy transfer. J Biol Chem 276:48040–48047.

Cheng ZJ, Zhao J, Sun Y, Hu W, Wu YL, Cen B, Wu GX, Pei G (2000): Beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. J Biol Chem 275:2479–2485.

Conlan LA, Martin TJ, Gillespie MT (2002): The COOH-terminus of parathyroid hormone-related protein (PTHrP) interacts with beta-arrestin 1B. FEBS Lett 527:71–75.

Couve A, Kittler JT, Uren JM, Calver AR, Pangalos MN, Walsh FS, Moss SJ (2001): Association of GABA(B) receptors and members of the 14–3–3 family of signaling proteins. Mol Cell Neurosci 17:317–328.

Cvejic S, Devi LA (1997): Dimerization of the delta opioid receptor: Implication for a role in receptor internalization. J Biol Chem 272:26959–26964.

Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ (1999): Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. J Biol Chem 274:17058–17062.

Dev KK, Nakajima Y, Kitano J, Braithwaite SP, Henley JM, Nakanishi S (2000): PICK1 interacts with and regulates PKC phosphorylation of mGLUR7. J Neurosci 20:7252–7257.

Duchene J, Schanstra JP, Pecher C, Pizard A, Susini C, Esteve JP, Bascands JL, Girolami JP (2002): A novel protein–protein interaction between a G protein-coupled receptor and the phosphatase SHP-2 is involved in bradykinin-induced inhibition of cell proliferation. J Biol Chem 277:40375–40383.

Elmhurst JL, Xie Z, O'Dowd BF, George SR (2000): The splice variant D3nf reduces ligand binding to the D3 dopamine receptor: Evidence for heterooligomerization. Brain Res Mol Brain Res 80:63–74.

Fraser ID, Cong M, Kim J, Rollins EN, Daaka Y, Lefkowitz RJ, Scott JD (2000): Assembly of an A kinase-anchoring protein-beta(2)-adrenergic receptor complex facilitates receptor phosphorylation and signaling. Curr Biol 10:409–412.

George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF (2000): Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem 275:26128–26135.

Georgoussi Z, Milligan G, Zioudrou C (1995): Immunoprecipitation of opioid receptor-Go-protein complexes using specific GTP-binding-protein antisera. Biochem J 306(Pt 1):71–75.

Gines S, Hillion J, Torvinen M, Le Crom S, Casado V, Canela EI, Rondin S, Lew JY, Watson S, Zoli M, Agnati LF, Verniera P, Lluis C, Ferre S, Fuxe K, Franco R (2000): Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci USA 97:8606–8611.

He J, Xu J, Castleberry AM, Lau AG, Hall RA (2002): Glycosylation of beta(1)-adrenergic receptors regulates receptor surface expression and dimerization. Biochem Biophys Res Commun 297:565–572.

Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C, Bouvier M (1996): A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J Biol Chem 271:16384–16392.

Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibanez CF, Lluis C, Franco R, Ferre S, Fuxe K (2002): Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. J Biol Chem 277:18091–18097.

Hu LA, Tang Y, Miller WE, Cong M, Lau AG, Lefkowitz RJ, Hall RA (2000): Beta 1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of beta 1-adrenergic receptor interaction with *N*-methyl-D-aspartate receptors. J Biol Chem 275:38659–38666.

Jordan BA, Devi LA (1999): G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399:697–700.

Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA (2001): Oligomerization of opioid receptors with beta 2-adrenergic receptors: A role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci U S A 98:343–348.

Ju H, Venema VJ, Marrero MB, Venema RC (1998): Inhibitory interactions of the bradykinin B2 receptor with endothelial nitric-oxide synthase. J Biol Chem 273:24025–24029.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998): GABA(B)-receptor subtypes assemble into functional heteromeric complexes. Nature 396:683–687.

Kishi H, Krishnamurthy H, Galet C, Bhaskaran RS, Ascoli M (2002): Identification of a short linear sequence present in the C-terminal tail of the rat follitropin receptor that modulates arrestin–3 binding in a phosphorylation-independent fashion. J Biol Chem 277:21939–21946.

Kitano J, Kimura K, Yamazaki Y, Soda T, Shigemoto R, Nakajima Y, Nakanishi S (2002): Tamalin, a PDZ domain-containing protein, links a protein complex formation of group 1 metabotropic glutamate receptors and the guanine nucleotide exchange factor cytohesins. J Neurosci 22:1280–1289.

Klein U, Ramirez MT, Kobilka BK, von Zastrow M (1997): A novel interaction between adrenergic receptors and the alpha-subunit of eukaryotic initiation factor 2B. J Biol Chem 272:19099–19102.

Latif R, Graves P, Davies TF (2001): Oligomerization of the human thyrotropin receptor: Fluorescent protein-tagged hTSHR reveals post-translational complexes. J Biol Chem 276:45217–45224.

Lavoie C, Mercier JF, Salahpour A, Umapathy D, Breit A, Villeneuve LR, Zhu WZ, Xiao RP, Lakatta EG, Bouvier M, Hebert TE (2002): Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy. J Biol Chem 277:35402–35410.

Law SF, Reisine T (1992): Agonist binding to rat brain somatostatin receptors alters the interaction of the receptors with guanine nucleotide-binding regulatory proteins. Mol Pharmacol 42:398–402.

Law SF, Manning D, Reisine T (1991): Identification of the subunits of GTP-binding proteins coupled to somatostatin receptors. J Biol Chem 266:17885–17897.

le Maire M, Champeil P, Moller JV (2000): Interaction of membrane proteins and lipids with solubilizing detergents. Biochim Biophys Acta 1508:86–111.

Lin R, Karpa K, Kabbani N, Goldman-Rakic P, Levenson R (2001a): Dopamine D2 and D3 receptors are linked to the actin cytoskeleton via interaction with filamin A. Proc Natl Acad Sci USA 98:5258–5263.

Lin SH, Arai AC, Wang Z, Nothacker HP, Civelli O (2001b): The carboxyl terminus of the prolactin-releasing peptide receptor interacts with PDZ domain proteins involved in alpha-amino-3-hydroxy-methylisoxazole-4-propionic acid receptor clustering. Mol Pharmacol 60:916–923.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999): Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283:655–661.

Matesic DF, Manning DR, Wolfe BB, Luthin GR (1989): Pharmacological and biochemical characterization of complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein. J Biol Chem 264:21638–21645.

Matesic DF, Manning DR, Luthin GR (1991): Tissue-dependent association of muscarinic acetylcholine receptors with guanine nucleotide-binding regulatory proteins. Mol Pharmacol 40:347–353.

Okuma Y, Reisine T (1992): Immunoprecipitation of alpha 2a-adrenergic receptor-GTP-binding protein complexes using GTP-binding protein selective antisera. Changes in receptor/GTP-binding protein interaction following agonist binding. J Biol Chem 267:14826–14831.

Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ (2002): Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. Science 298:834–836.

Pfeiffer M, Koch T, Schroder H, Klutzny M, Kirscht S, Kreienkamp HJ, Hollt V, Schulz S (2001): Homo- and heterodimerization of somatostatin receptor subtypes. Inactivation of sst(3) receptor function by heterodimerization with sst(2A). J Biol Chem 276:14027–14036.

Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, Schulz S (2002): Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem 277:19762–19772.

Richman JG, Brady AE, Wang Q, Hensel JL, Colbran RJ, Limbird LE (2001): Agonist-regulated interaction between alpha2-adrenergic receptors and spinophilin. J Biol Chem 276:15003–15008.

Romano C, Yang WL, O'Malley KL (1996): Metabotropic glutamate receptor 5 is a disulfide-linked dimer. J Biol Chem 271:28612–28616.

Salim K, Fenton T, Bacha J, Urien-Rodriguez H, Bonnert T, Skynner HA, Watts E, Kerby J, Heald A, Beer M, McAllister G, Guest PC (2002): Oligomerization of G-protein-coupled receptors shown by selective co-immunoprecipitation. J Biol Chem 277:15482–15485.

Sanchez C, Rueda D, Segui B, Galve-Roperh I, Levade T, Guzman M (2001): The CB(1) cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein fan. Mol Pharmacol 59:955–959.

Shih M, Lin F, Scott JD, Wang HY, Malbon CC (1999): Dynamic complexes of beta2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. J Biol Chem 274:1588–1595.

Sidhu A, Kimura K, Uh M, White BH, Patel S (1998): Multiple coupling of human D5 dopamine receptors to guanine nucleotide binding proteins Gs and Gz. J Neurochem 70:2459–2467.

Suzuki S, Chuang LF, Yau P, Doi RH, Chuang RY (2002): Interactions of opioid and chemokine receptors: Oligomerization of mu, kappa, and delta with CCR5 on immune cells. Exp Cell Res 280:192–200.

Tardif M, Brouchon L, Rabiet MJ, Boulay F (2003): Direct binding of a fragment of the Wiskott-Aldrich syndrome protein to the C-terminal end of the anaphylatoxin C5a receptor. Biochem J 372:453–463.

Xia Z, Gray JA, Compton-Toth BA, Roth BL (2003): A direct interaction of PSD-95 with 5-HT2A serotonin receptors regulates receptor trafficking and signal transduction. J Biol Chem 278:21901–21908.

Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ, Worley PF (1998): Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. Neuron 21:707–716.

Xu XZ, Choudhury A, Li X, Montell C (1998): Coordination of an array of signaling proteins through homo- and heteromeric interactions between PDZ domains and target proteins. J Cell Biol 142:545–555.

Xu J, Paquet M, Lau AG, Wood JD, Ross CA, Hall RA (2001): Beta 1-adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted–2 (MAGI–2). Differential regulation of receptor internalization by MAGI–2 and PSD-95. J Biol Chem 276:41310–41317.

Xu J, He J, Castleberry AM, Balasubramanian S, Lau AG, Hall RA (2003): Heterodimerization of alpha 2A- and beta 1-adrenergic receptors. J Biol Chem 278:10770–10777.

Yoshioka K, Saitoh O, Nakata H (2001): Heteromeric association creates a P2Y-like adenosine receptor. Proc Natl Acad Sci USA 98:7617–7622.

Zeng FY, Wess J (1999): Identification and molecular characterization of m3 muscarinic receptor dimers. J Biol Chem 274:19487–19497.

Zitzer H, Richter D, Kreienkamp HJ (1999): Agonist-dependent interaction of the rat somatostatin receptor subtype 2 with cortactin-binding protein 1. J Biol Chem 274:18153–18156.