Identification of Novel G Protein-Coupled Receptor-Interacting Proteins.

Richard T. Premont†, and Randy A. Hall‡

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† Liver Center, Department of Medicine (Gastroenterology), Box 3083, Duke University Medical Center, Durham, NC 27710.

‡ Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322

Running header: GPCR-Interacting Proteins

The superfamily of G protein-coupled receptors includes proteins that recognize and respond to ligands or agonists as diverse as proteins, peptides, small molecules, ions, and photons. To accommodate this diversity of activators, the G protein-coupled receptor superfamily comprises one of the largest gene families known. These receptor proteins share a conserved seven-transmembrane span structure, but can be classified into several distinct groups or families each with unique conserved sequence elements (1).

Physiological responses to individual agonists are initiated by the binding to specific G protein-coupled receptor proteins on the cell surface. Binding of the agonist to the receptor leads to an activated state that is capable of serving as a guanine nucleotide exchange factor for specific heterotrimeric G proteins (2). Like all GTP-binding proteins, heterotrimeric G proteins (or more precisely, their -subunits) are active when bound to GTP and inactive when bound to GDP (3). In the inactive state, each GDP-bound -subunit is associated with a -subunit complex. Activated receptors both promote the release of GDP from the G protein -subunit and catalyze the binding of GTP in its place. Once activated through receptor-catalyzed GDP-GTP exchange, the heterotrimeric G protein undergoes subunit dissociation.

The GTP-bound -subunit and the freed -subunit complex each interact with and modulate the activity of intracellular messenger-generating proteins, or G protein effectors (4). For example, the Gs -subunit activates adenylyl cyclase to produce cyclic AMP. Specific G protein subtypes and subsequent effector pathways that can be activated by a particular receptor are said to be 'coupled' to that receptor. Individual receptor types may couple to a single G protein subtype, or to several related or even unrelated G proteins. Individual G protein subtypes activate only a specific set of effectors, which then mediate some subset of the cellular effects initiated by receptor activation. The simultaneous activation of all the G protein effectors coupled to a particular receptor is presumed to give a unique signature that determines the response of the cell to activation by that particular receptor agonist.

G protein-coupled receptor systems are not static, but quickly adapt to the activity state of the cell they reside in. The loss of responsiveness following prolonged or repeated activation is called desensitization (5). Desensitizing events that affect signaling pathways in addition to that which provoked the desensitization are termed 'heterologous', and may include regulation of receptors as well as other components of the signal transduction cascades. Desensitizing events that affect only the signaling ability of the agonist that provoked the desensitization are termed 'homologous', and primarily involve regulation of that individual receptor subtype. One major form of receptor activation-dependent or homologous desensitization is the uncoupling of the receptor from the ability to activate G proteins. Numerous studies in several receptor systems have shown that receptor uncoupling is coincident with receptor protein phosphorylation (5).

A family of G protein-coupled receptor kinases, or GRKs, have been described that strongly prefer the activated or agonist-occupied form of the receptor as a phosphorylation substrate (6,7). Due to this activation dependence, GRKs serve initiate the uncoupling of activated G protein-coupled receptors. Two retinal GRKs serve as rhodopsin and cone opsin kinases (GRK1 and GRK7, respectively), while five somatic GRKs (GRKs 2-6) appear to serve to regulate all other G protein-coupled receptors in the body. Receptor phosphorylation in itself does not appear to alter receptor function greatly, but rather targets the activated receptors for

uncoupling. The actual uncoupling event requires the action of an additional protein family, the arrestins. Arrestin proteins bind to the GRK-phosphorylated receptor proteins and prevent activated receptors from further coupling to or activating G proteins (8).

Thus, there are three classical G protein-coupled receptor-interacting proteins: the heterotrimeric G proteins, the GRKs, and the arrestins. All three appear to recognize a multitude of distinct receptor subtypes, and all prefer the agonist-occupied, activated conformation of the receptor. Amongst these proteins, the general mechanisms of receptor signaling to G proteins and homologous desensitization are accounted for.

The G protein paradigm has been validated innumerable times, for a great variety of receptor types. Nonetheless, the fact that individual receptor subtypes exhibit unique signaling properties has re-emerged as an important issue after a period of dormancy during which the generality of signaling was the primary focus (9). That is, many receptor types do not behave quite as expected based on the reductionist assumption that any receptor types coupled to the same set of G proteins should be interchangeable. Since receptors do exhibit such differences, the question has been asked whether there might exist novel proteins that interact with individual receptors or subsets of receptors. Such receptor-interacting proteins might *i*) serve direct signaling roles, *ii*) localize a receptor to a particular region on the cell surface, *iii*) scaffold other defined signaling molecules in association with a receptor, or *iv*) assist in targeting the receptor as it cycles from cell surface to intracellular membranes following activation.

The earliest-known GPCR-interacting proteins, the G proteins, GRKs and arrestins, were first purified based on their physiological activities, and their direct association with receptors was demonstrated much later. Similarly, the receptor activity-modifying proteins (RAMPs) were cloned based on functional studies demonstrating that they are required for proper expression and activity of receptors for adrenomedullin, amylin and calcitonin-gene related peptide (10), and only following this identification in functional screens were the RAMPs shown to associate directly with the receptors they modulate. In one case, for the $GABA_B$ receptor, the dimeric interaction of two distinct, related 7TM proteins is required for receptor expression and function (11). The converse approach to finding GPCR-interacting proteins, *i.e.* identification of a protein that directly associates with a given GPCR, followed by functional studies to address the physiological significance of the interaction, has also proven useful in understanding GPCR signaling and regulation. This approach has been particularly useful in identifying cytoplasmic proteins that associate specifically with one or several GPCR subtypes through interaction with subtype-specific structural motifs.

Within the family of G protein-coupled receptors, the highest sequence and function conservation is associated with the ligand binding and G protein coupling regions of the membrane spans and juxtamembrane portions of the intracellular loops and carboxyl terminal tail. G protein coupled receptors exhibit low sequence conservation in the intracellular loops and carboxyl terminal tail regions further from the membrane. Integral membrane receptor proteins are notoriously difficult to work with, so using an intact and active receptor protein as a screening tool has been impractical. However, these more unique loop and tail regions can be used as bait to search for novel receptor-interacting proteins using a variety of biochemical techniques.

Receptor fragment-fusion protein affinity chromatography:

One approach to identify potential receptor binding proteins is the biochemical one of using association with the receptor itself as a form of affinity chromatography. This could be accomplished by immunoprecipitating the full-length receptor protein and searching for co-immunoprecipitated protein bands. Generally low endogenous receptor expression and lack of adequate anti-receptor antibodies often necessitates transfection of epitope-tagged receptors. Further, the interacting proteins of interest may not be present in readily available cell lines, and there can be difficulties in scaling up such an assay. Nonetheless, co-immunoprecipitation has been used in several cases to identify the physical receptor interaction of proteins known to be functionally linked to a given receptor, for example the interaction of endothelial nitric oxide synthase with the bradykinin B2 receptor (12) and the interaction of the small GTP-binding proteins ARF and Rho with several GPCRs (13). The co-immunoprecipitation approach is typically limited, however, to studies aimed at confirming the potential interaction of suspected GPCR binding partners rather than screens for novel and unsuspected GPCR-interacting proteins.

A method related to co-immunoprecipitation but more useful for large-scale screening efforts is affinity chromatography utilizing immobilized receptor fragments as the bait. This method is technically simple, scalable and adaptable. The preparation and immobilization of the receptor fragment can be achieved by any of several methods, and tissue or cell lysates from any source and in any amount can be applied. However, this technique may not be amenable to detecting protein interactions that require recognition of overall receptor conformations (eg., ligand-activated state). Fusion protein affinity chromatography has been used successfully to identify a number of GPCR interactions with cytoplasmic proteins, including the interaction of calmodulin with the metabotropic glutamate receptor type 5 (14), the interaction of the Na+H+ exchanger regulatory factor with the $_2$-adrenergic receptor (15), the interaction of Grb2 and Nck with the dopamine D4 receptor (16), the interaction of SH3p4/endophilin-1 with the $_1$-adrenergic receptor (17), and the interaction of 14-3-3 proteins with the $_2$-adrenergic receptors (18). Here we discuss the use of glutathione *S*-transferase (GST) fusion proteins as the affinity bait (19,20), as utilized in studies aimed at identifying novel -adrenergic receptor-interacting proteins.

The receptor fragment of interest is prepared by polymerase chain reaction using specific oligonucleotide primers. For the human $_1$- and $_2$-adrenergic receptors, third intracellular loop and carboxyl terminal tail fusions originally created for use as antigens for antisera production were used. Briefly, receptor fragments are amplified using specific oligonucleotide primers encoding the relevant receptor domains, using the receptor cDNA as template. Sense primers contain *BamHI* restriction sites and antisense primers *EcoRI* sites for subcloning of the amplified fragments in the pGEX-2TK vector (Amersham Pharmacia). Subcloned inserts are analyzed for the proper DNA sequence. Plasmids bearing the desired fusion inserts are used to transform competent *Escherichia coli* cells for expression. For pGEX series vectors, the BL21 strain of *E. coli* is a suitable host cell. From an overnight culture, two ml are used to inoculate 1 liter of LB media containing 100 µg/ml ampicillin, and the cells allowed to grow at 37° until the culture reaches an A_{600} of 0.6 (approximately 3 h). At this time, expression of the fusion protein is induced by addition of IPTG to 500 mM final concentration, and the cells are grown for an

additional 2 h at 37°. Induced bacterial cells are collected by centrifugation at 5,000xg in a Sorvall HBB6 rotor for 10 min at 4°. The induced cell pellet is resuspended in 30 ml per liter of original culture volume with phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (5 µg/ml aprotinin, 150 µg/ml benzamidine, 5 µg/ml leupeptin, 4 µg/ml pepstatin, and 20 µg/ml PMSF). After the cells are resuspended, 1 ml of freshly prepared 1 mg/ml lysozyme solution in PBS is added and mixed gently. The resuspended cells are flash-frozen in liquid nitrogen or dry ice-acetone, and stored frozen at –80° until needed.

GST fusion proteins are purified from induced bacterial cell lysates using affinity chromatography on glutathione-agarose (20) (Sigma). Frozen cells are thawed gently and, once thawed, allowed to sit at 4° for 15 min for the lysozyme to digest the cell wall. The lysates are spun at 20,000xg in Sorvall SS-34 rotor for 30 min at 4°, and the clarified supernatant transferred to a 50 ml screw-cap tube. For each liter of original cell culture, 1 ml of a 50% slurry of glutathione-agarose beads is added, and the tube rotated for 2 h at 4°. The beads are spun at 2,000xg for 5 min at 4°, and washed with 40 ml of cold PBS, three times. At this point, beads containing bound GST-fusion protein can be used for pull down assays, and the quality and quantity of the bound fusion protein assessed by SDS-PAGE and Coomassie blue staining. Alternatively, for studies where the GST-fusion is required free of glutathione-agarose beads, the fusion protein is eluted by addition of 10 ml of PBS containing 1 mM glutathione and protease inhibitors. The supernatant containing eluted fusion protein is removed from the beads following centrifugation. If needed, the glutathione and/or protease inhibitors can be removed by several repeated cycles of concentration in CentriPrep 30 spin concentrators (Amicon) and dilution with PBS buffer.

Lysates prepared from tissue can contain only soluble proteins (in the absence of detergent) or a mixture of soluble and membrane-associated proteins (extraction with detergent), or may be prepared from just the membrane fraction using detergent extraction. Since separating the soluble and membrane-associated proteins before any affinity chromatography can provide a significant purification in its own right, this may be a favorable approach. From a bovine brain, both types of lysates are easily prepared. First, the brain is minced into roughly 1 cm cubes and crudely homogenized in a blendor using 5 volumes of homogenization buffer: 20 mM HEPES (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors. The homogenate is then more finely disrupted in small aliquots using a large bore Polytron for 1 minute. The homogenate is spun at 50,000xg for 30 min at 4°, and the resulting supernatant pooled for use as the 'soluble lysate'. To prepare membranes, the pellets are homogenized again with the Polytron in the original volume of homogenization buffer and spun at 1,000xg for 10 min at 4° to remove remaining particulates, and the resulting supernatant is then spun at 20,000xg for 30 min at 4°. After decanting, the membrane pellets are resuspended using a Polytron in 10 volumes of homogenization buffer, which is then supplemented with the desired detergent for extraction, such as 1% NP-40, Triton X-100 or CHAPS. After rotating or stirring for 1 h at 4° to extract membrane proteins, the lysate is spun at 50,000xg for 1h at 4°. The resulting supernatant is pooled as the 'membrane lysate'.

To identify receptor fragment-interacting proteins in tissues or cells of interest, a lysate prepared from that tissue is mixed with glutathione-agarose beads still bound to the GST-receptor fragment fusion protein. In the case of the $_1$- and $_2$-adrenergic receptor carboxyl

terminal tails, bovine brain, heart and kidney membrane lysates prepared using 1% CHAPS are added to the fusion protein beads, or to beads bound to GST bearing no fusion, and rotated for 1 h at 4°. The beads are washed five times batchwise with homogenization buffer, and bound proteins eluted in SDS-PAGE sample buffer. SDS-PAGE electrophoresis and Coomassie blue staining reveals that a single 50-kDa protein band in samples containing both kidney lysate and the GST- $_2$-adrenergic receptor tail fusion beads (Figure 1). This protein, upon microsequencing, was identified as the NHERF protein, which specifically recognizes the carboxyl terminal DSLL motif of the $_2$-adrenergic receptor through a PDZ domain-mediated interaction (15). In the case of the $_1$- and $_2$-adrenergic receptor third intracellular loops, bovine brain soluble lysate is added to the fusion protein beads, or to beads bound to GST bearing no fusion, and rotated for 1 h at 4°. The beads are washed five times batchwise with homogenization buffer, and bound proteins eluted in SDS-PAGE sample buffer. SDS-PAGE electrophoresis and Coomassie blue staining reveals that a single 40-kDa protein band in samples containing both brain lysate and the GST- $_1$-adrenergic receptor third intracellular loop fusion beads (Figure 2). This protein, upon microsequencing, was identified as the SH3p4/endophilin 1 protein, which specifically recognizes a polyproline motif in the third intracellular loop of the $_1$-adrenergic receptor through an SH3 domain-mediated interaction (17).

Receptor fragment-fusion protein overlay:

The ability to purify a receptor fragment-interacting protein to sufficient purity and in sufficient quantity to allow direct microsequencing requires a combination of factors. First, the receptor fragment used as the bait must be of sufficient purity that impurities in the preparation itself do not mask any purified proteins. Some fusion proteins may be unstable, and new constructs with slightly altered fusion boundaries may prove more tractable. Second, binding and washing conditions may affect the ability of the fusion to bind to interacting proteins. This is particularly notable for membrane lysates, where the choice of detergent may be critical to efficient extraction and to subsequent binding. Finally, the choice of tissue dictates the quantity of interacting protein available to be purified, and if present at too low a level, it may not be detected by Coomassie blue staining. Alternative labeling methods, such as silver staining or trace iodination, may be needed, or cells may be labeled with radioactive amino acids prior to preparation of the lysate.

One distinct approach that may help in detect receptor-interacting proteins is to use the affinity of the receptor fragment itself as part of the detection system, such as by ‘far western’ or protein overlay blotting. In this technique, samples to be probed, either tissue lysates or proteins that have bound to immobilized receptor fragment fusions as described above, are separated by SDS-PAGE and transferred to a nitrocellulose or Nylon membrane. Potential interacting proteins are then detected by first allowing the soluble receptor fragment to bind to proteins on the membrane, and then detecting where the soluble fragment has bound to the filter. Detection can be immunological, or the probe receptor fragment can be radiolabelled prior to adding it to the membrane. The advantages of the blot overlay approach are that it can be quite sensitive and that it allows for examination of many tissues at one time. The main disadvantage is that the proteins on the blot are denatured and conformationally restrained, and this may inhibit many protein-protein interactions. Another disadvantage is that detection of a GPCR-interacting protein via this blot overlay does not automatically lead to identification of the interacting

protein. However, blot overlays can be effectively combined with receptor fragment affinity chromatography, as described above, to find and then identify GPCR-interacting proteins.

An example of a blot overlay experiment is shown in Figure 3. The probe in this case is prepared from GST- $_2$-adrenergic receptor carboxyl terminal tail protein by phosphorylation *in vitro*. The pGEX 2TK vector encodes GST fusions with a protein kinase A site immediately prior to the fusion protein but immediately after a thrombin cleavage site. The glutathione-agarose bead-bound fusion protein (100 μg in 1 ml) is incubated with protein kinase A catalytic subunit (Promega) and 100 μCi of [-^{32}P]ATP for 30 min at 30°. The beads are washed with PBS in the absence of protease inhibitors, and the radiolabeled receptor fragment removed from the GST and beads by cleavage with thrombin (Novagen Thrombin cleavage kit). The resulting free ^{32}P-labeled $_2$-adrenergic receptor carboxyl terminal tail is then added to the nitrocellulose filter in PBS containing 2% milk and 0.1% Tween-20, and incubated at room temperature with shaking for 1 h. The blot is washed several times for 5 min with the same buffer lacking radioactive probe, and exposed to X-ray film. The radiolabeled receptor fragment specifically binds to a 50-kDa band that is highly enriched in kidney tissue but not detectable in heart, brain or liver tissue. These overlay studies led to the use of kidney tissue as a starting material for the receptor fragment affinity chromatography studies illustrated in Figure 1, which led ultimately to the identification of NHERF as a high-affinity binding partner of the $_2$-adrenergic receptor.

Receptor fragment 2 hybrid screening:

As noted above, biochemical approaches to identifying receptor interacting proteins require that the target protein exists in sufficient abundance to be identified after being partially purified. However, individual G protein-coupled receptors themselves are often expressed at quite low levels, on the order of fmol/mg cell protein. A protein that interacts with one particular receptor subtype may be present at quite low levels in tissues compared to G proteins or arrestins, which are present at quantities on scale more with the *sum* of all receptors present. In this case, biochemical approaches may fail to detect important regulatory interactions. A complementary approach is to use genetic screening for protein-protein interactions, such as the two-hybrid or interaction-trap method. Briefly, this technique involves creating a fusion protein between a GPCR fragment and the DNA binding domain of a transcription factor (GAL4, for example). This fusion protein is then co-transformed into yeast cells with a library of random cDNAs fused to the activation domain of the transcription factor. Interactions between the GPCR fragment and other proteins are detected when the DNA binding domain and activation domain are brought together, thereby inducing expression of a reporter gene. The general technical details of two-hybrid screening have been described previously in this series (21), and required reagents are generally available commercially as kits (*e.g.*, Clontech Matchmaker system), so we will limit the discussion here to particular caveats in screening for receptor-interacting proteins.

There are a few important considerations in applying the yeast two-hybrid system to screens for GPCR-interacting proteins. First of all, since the fusion proteins must be translocated to the nuclei of the yeast in order to turn on the reporter genes, a piece of the receptor must be chosen which can exist as a stable, soluble fusion protein. Fusion proteins containing a transmembrane region are unlikely to yield positive results, since the fusion protein probably will

not fold properly and may be inserted into the plasma membrane or retained in the endoplasmic reticulum. Secondly, interactions requiring post-translational modification of a GPCR (such as phosphorylation) are unlikely to be detected via the yeast two-hybrid system. Thirdly, interactions requiring non-contiguous epitopes or the global conformation of a GPCR will almost certainly not be detected in two-hybrid screens with GPCR fragments. In spite of these limitations, yeast two-hybrid screening has proven successful in identifying a number of GPCR interactions that have subsequently been shown to occur in mammalian cells, including the interaction of the eukaryotic initiation factor 2B with adrenergic receptors (22), the immediate early gene Homer with metabotropic glutamate receptors (23), the motor protein dynein with rhodopsin (24), cortactin-binding protein 1 with the somatostatin receptor type 2 (25), SH3p4/endophilin-1 with the $_1$-adrenergic receptor (17), the transmembrane protein calcyon with the dopamine D1 receptor (26), and the PDZ protein PICK1 with the metabotropic glutamate receptor type 7 (27).

Summary

Biochemical and genetic methods utilizing G protein-coupled receptor fragments have been used successfully to identify G protein-coupled receptor-interacting proteins. As noted above, these methods may be unable to detect interactions that require important conformations of the native receptor protein, but have nevertheless proven quite useful in expanding our understanding of receptor regulation to include interactions with proteins other than G proteins, G protein-coupled receptor kinases and arrestins. Undoubtedly, other G protein-coupled receptors also have their own particular constellations of associated proteins, and the techniques described here should prove useful in identifying these.

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