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Proteomic analysis of native metabotropic glutamate receptor 5 protein complexes reveals novel molecular constituents

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Abstract

We used a proteomic approach to identify novel proteins that may regulate metabotropic glutamate receptor 5 (mGluR5) responses by direct or indirect protein interactions. This approach does not rely on the heterologous expression of proteins and offers the advantage of identifying protein interactions in a native environment. The mGluR5 protein was immunoprecipitated from rat brain lysates; co-immunoprecipitating proteins were analyzed by mass spectrometry and identified peptides were matched to protein databases to determine the correlating parent proteins. This proteomic approach revealed the interaction of mGluR5 with known regulatory proteins, as well as novel proteins that reflect previously unidentified molecular constituents of the mGluR5-signaling complex. Immunoblot analysis confirmed the interaction of high confidence proteins, such as phosphofurin acidic cluster sorting protein 1, microtubule-associated protein 2a and dynamin 1, as mGluR5-interacting proteins. These studies show that a proteomic approach can be used to identify candidate interacting proteins. This approach may be particularly useful for neurobiology applications where distinct protein interactions within a signaling complex can dramatically alter the outcome of the response to neurotransmitter release, or the disruption of normal protein interactions can lead to severe neurological and psychiatric disorders.

Keywords

mass spectrometry; metabotropic glutamate receptor; protein interaction; proteomics

Glutamate is the principal excitatory amino acid neurotransmitter in the central nervous system (CNS) and activates two distinct families of glutamate receptors (GluRs). The ionotropic glutamate receptor (iGluR) family is composed of *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors that mediate fast synaptic transmission, while the metabotropic glutamate receptors (mGluRs) modulate cell excitability and synaptic transmission. Group I mGluRs (1 and 5) couple to the Gq signaling pathway, whereas Group II mGluRs (2 and 3) and Group III mGluRs (4, 6, 7 and 8) couple to Gi/Go signaling pathways in heterologous expression systems (Conn and Pin

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1997). mGluR signaling is regulated by several mechanisms, including direct and indirect protein interactions, and the goal of this study was to identify novel regulatory proteins that are molecular constituents of the mGluR5-signaling complex. mGluR5 is expressed in postsynaptic (Romano *et al*. 1995; Shigemoto *et al*. 1997) and presynaptic membranes (Gereau and Conn 1995; Croucher *et al*. 2001), interneurons (van Hooft *et al*. 2000), glial cells (van den Pol *et al*. 1995) and, more recently, in nuclei where they may mediate intranuclear signaling pathways (O'Malley *et al*. 2003). This diverse distribution is consistent with the role of mGluR5 in the control of an array of key signaling events, including roles in the adaptive changes needed for long-term depression or potentiation of neuronal synaptic connectivity (for review see Hermans and Challiss 2001). In addition to playing critical physiological roles within the brain, Group I mGluRs are of considerable importance because of their potential as drug targets for the treatment of a variety of neurological and psychiatric disorders.

Several studies have defined a major site for regulation of mGluR signaling at the intracellular carboxy terminal domain (CTD) that, together with the second intracellular domain, comprise the G protein-binding site (Pin *et al*. 1994). For example, phosphorylation of the CTD by protein kinase C (PKC) acts in concert with NMDA-mediated calcineurin dephosphorylation of the CTD to regulate Group I mGluR signaling in neurons (Gereau and Heinemann 1998; Alagarsamy *et al*. 1999). Several proteins bind directly to the CTD of Group I mGluRs to regulate signaling, including Homer (Brakeman *et al*. 1997; Tu *et al*. 1998), the G proteins Gq alpha (Abe *et al*. 1992) and Go alpha (McCool *et al*. 1998), calmodulin (Minakami *et al*. 1997), beta-tubulin (Ciruela *et al*. 1999), Siah1A (Ishikawa *et al*. 1999) and protein phosphatase 1C (Croci *et al*. 2003). In addition, previous data support an indirect interaction between mGluR5 and the inositol trisphosphate receptor (IP3R) (Tu *et al*. 1998; Kammermeier *et al*. 2000), phospholipase C (PLC) beta (Kim *et al*. 1997), Shank (Ehlers 1999), arrestin (Mundell *et al*. 2001) and Src-family protein tyrosine kinases (Heuss *et al*. 1999). The functional roles of mGluRs within a cell may reflect their unique interactions with regulatory proteins within a signaling complex. For example, mGluR5 and mGluR1 are co-expressed in the same neuron yet mediate distinct physiological responses (Valenti *et al*. 2002; Pellegrini-Giampietro 2003). Classical approaches to study specific protein interactions include yeast two-hybrid screens or the use of glutathione-*S*-transferase (GST)-fusion proteins to identify interacting proteins. However, these approaches may not detect ternary or weak protein interactions, or protein interactions that are mediated by post-translational modifications such as lipid modification (Ashman *et al*. 2001) or the phosphorylation/dephosphorylation state of the protein (Slepnev *et al*. 1998). Thus, to identify novel mGluR5-interacting proteins, we utilized a proteomic approach that does not rely on the heterologous expression of proteins and offers the advantage of identifying protein interactions in a native environment.

The term ‘proteome’ was coined in 1995 (Wasinger *et al*. 1995) and defines the large-scale characterization of the entire protein complement of a cell line, tissue or organism. Proteomic studies have expanded greatly in the last decade, largely due to two significant scientific advancements: (i) increased sensitivity of analysis and accuracy of results for mass spectrometry protein identification such that proteins in the femtomolar range can now be detected; and (ii) advances in large-scale nucleotide sequencing of expressed sequence tags and genomic DNA that provides necessary information for protein identification. The first use of a proteomic approach to identify neuronal protein–protein interactions focused on the NMDA receptor complex (Husi *et al*. 2000). This study included an analysis by mass spectrometry of proteins that were co-immunoprecipitated with the NMDA receptor; the experiments revealed the identity of (at least) 77 proteins ranging from receptors to adaptors, signaling molecules and cytoskeletal proteins, as well as several novel proteins that comprise the NMDA receptor complex. Similar proteomic studies have been used to identify major proteins in the postsynaptic density (PSD) (Walikonis *et al*. 2000), novel proteins that interact with the purinergic P2X receptor (Kim *et al*. 2001) and membrane proteins that interact with

the CTD of the serotonin 5-HT(2C) receptor (Bécamel *et al*. 2002). To identify proteins that interact with mGluR5, we immunoprecipitated the receptor from rat brain lysates, subjected the samples to liquid chromatography and mass spectrometry, and identified the protein by comparing the resulting data with protein databases. This approach revealed the interaction of mGluR5 with known regulatory proteins, as well as novel interacting proteins that may reflect previously unidentified molecular constituents of the mGluR5-signaling complex.

Materials and methods

Antibodies

The following primary antibodies were used in these experiments: rabbit anti-mGluR1a and rabbit anti-mGluR5 (Upstate, Lake Placid, NY, USA), mouse anti-PACS1a (phosphofurin acidic cluster sorting protein 1; BD Transduction Laboratories, Lexington, KY, USA), mouse anti-MAP2 (microtubule-associated protein 2; Sigma, St Louis, MO, USA) and mouse anti-dynamin (Upstate). All secondary antibodies were obtained from Bio-Rad (Hercules, CA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Cascade Blue (CB; Molecular Probes, Eugene, OR, USA) was used as the negative control antibody in all of these experiments as the mGluR5 and the CB antibody are both rabbit IgG antibodies. CB hydrazide is an analog of the bright blue fluorescent tracer methoxypyrenetrisulfonic acid (Molecular Probes), and the rabbit anti-CB antibody was developed to characterize the morphology of neurons that have been filled with CB. As mammalian cells do not make CB, the proteins that are isolated by this antibody represent non-specific protein interactions with components used in the experimental protocol.

Brain lysate preparation

Frozen normal brains from adult male Sprague-Dawley rats (250–300 g) were purchased from Harlan Bioproducts (Indianapolis, IN, USA). For one experiment, adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were subjected to middle cerebral artery occlusion (MCAO) by the suture method for 30 min and reperfused for 24 h (Shimizu *et al*. 2001) to stimulate glutamate release *in vivo*. Each brain was homogenized in 10 mL cold lysis buffer [25 mM HEPES, 140 mM NaCl, 15 mM EGTA, 0.5% Triton X-100 and complete protease inhibitor (Roche, Basel, Switzerland) that includes chymotrypsin (1.5 μg/mL), thermolysin (0.8 μg/mL), papain (1 μg/mL), pronase (1.5 μg/mL), pancreatic extract (1.5 μg/mL) and trypsin (0.002 μg/mL)]. The homogenate was incubated on a rotary shaker for 30 min then centrifuged at 24 000 *g* for 15 min. The lysates were pre-cleared with 70 μL of a 1 : 1 (v/v) slurry of protein A-sepharose (PAS)/mL lysate and 3 μg of the CB antibody/mg lysate protein for 2 h, and then centrifuged again at 24 000 *g* for 15 min. The protein concentrations of the cleared lysates were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Co-immunoprecipitation

Individual tubes containing 60 μg primary antibody (experimental anti-mGluR5 or control anti-CB antibody for the immunoprecipitations; anti-Dyamin1, anti-MAP2 and anti-PACS1 for the reverse co-immunoprecipitations)/60 mg of lysate protein were incubated overnight at 4°C, and 100 μL of a 1 : 1 (v/v) PAS slurry were added to each tube and incubated for an additional 3 h with shaking. The tubes were spun at 800 *g* for 2 min, and the pellet was resuspended in wash buffer (25 mM HEPES, 140 mM NaCl, 15 mM EDTA, 0.1% Triton X-100) and centrifuged at 800 *g* for 2 min for a total of three washes; the residual supernatant fluid was removed from the PAS beads using a loading gel pipette tip.

Sample preparation

Two protocols were used to prepare the proteins that co-immunoprecipitated with mGluR5 and proteins that bound to the CB antibody, the negative control, for mass spectrometry. The proteins to be analyzed were either (i) removed *en masse* from the beads and digested with trypsin, or (ii) removed from the beads, separated by one-dimensional (1D) gel electrophoresis and digested with trypsin in gel. The first protocol consisted of *en masse* removal of the co-immunoprecipitated proteins from the PAS beads as indicated in the text and Tables 1–3 (#). Thus, the PAS bead–protein complex was resuspended twice in 30 μL citric acid (pH 2.0) at 37°C for 15 min to elute the immunoprecipitated proteins. The eluates were transferred to an Eppendorf tube and the pH was adjusted to 7.5 using 5 N NaOH. The samples were dialyzed into 0.1 M NH_4HCO_3 (pH 8), followed by the addition of urea to 2 M final concentration. The samples were incubated at 90°C for 20 min, returned to 37°C and brought to 10 mM dithiothreitol, and further incubated at 37°C for 1 h. The samples were then brought to 40 mM iodoacetamide, incubated in the dark at 37°C for 1 h and brought to 1 mM $CaCl_2$. At this point, the proteins were trypsinized by the addition of 2 μg trypsin (approximately 0.1 μg/μL final concentration) and incubated at 37°C for 24 h. Complete trypsin digestion was assessed by silver stained 1D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and if necessary, the trypsin digestion was repeated. Finally, the pH of the samples was lowered with trifluoroacetic acid (3% final concentration). The samples were then reduced to 100 μL in a speed vacuum and stored at − 20°C.

The *en masse* sample preparation protocol was modified because at times, the complexity of the sample was problematic for the mass spectrometry analysis. Thus, the proteins were removed from the beads and separated by 1D gel electrophoresis, as indicated in the text and Tables 1–3 (##). In the modified procedure, the PAS bead–protein complex was resuspended twice in 30 μL citric acid (pH 2.0) at 37°C for 15 min to elute the immunoprecipitated proteins. The eluates were transferred to an Eppendorf tube and the pH was adjusted to 7.5 using 5 N NaOH. At this point, SDS loading buffer was added to the samples [final concentration 1×: 0.0625 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% bromophenol blue] and separated by 1D gel electrophoresis on a 4–15% criterion gel (Bio-Rad). Protein visualization was obtained using either the PlusOne silver staining kit (Amersham Biosciences, Piscataway, NJ, USA) without glutardialdehyde, or the SilverQuest silver staining kit (Invitrogen Corporation, Carlsbad, CA, USA). The mGluR5 and the CB control lanes were each cut into 10 fragments, with the fragment regions from the mGluR5 matching the identical region in the CB control, and each individual fragment was subjected to mass spectrometry.

Immunoblot analysis

The proteins to be analyzed by immunoblot were removed from the beads and separated by 1D gel electrophoresis as described above (Sample preparation). The proteins in the gel were transferred to polyvinylidenedifluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA). The resultant protein blot was incubated with 5% (w/v) milk in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) for 1.5 h at room temperature, followed by incubation with the primary antibody diluted into 5% (w/v) milk in TBST for 1.5 h at room temperature. The blot was rinsed four times for 5 min each in TBST without milk and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) for 1 h at room temperature. The blot was rinsed once in TBST and four times in TBS, and the proteins were detected using an enhanced chemiluminescent (ECL) western blotting kit (Amersham Biosciences) and Kodak BioMax film (Eastman Kodak Co., Rochester, NY, USA).

Liquid chromatography (LC) and mass spectrometry (MS)

Two protocols at two proteomic facilities were used to identify the proteins that co-immunoprecipitated with mGluR5, and proteins that bound to the CB antibody, the negative control.

Protocol (A) Mass Spectrometry Laboratory, Oregon State University—All samples were mixed 1 : 1 (v/v) with solvent A [0.1% formic acid (FA), 0.005% trifluoroacetic acid (TFA) and 3% acetonitrile (ACN)], and 6 μL of this solution were injected onto a column. Solvent B contained 0.1% FA and 0.005% TFA in 80% ACN. A 5 mm × 0.32 mm C18 trap (LC Packing, San Francisco, CA, USA) was used to de-salt and concentrate each sample. A 10 cm long, 75 μm inner diameter PicoFrit column (New Objective, Woburn, MA, USA) was packed in-house with Jupiter C_{18} (Phenomenex, Inc., Torrance, CA, USA). The LC conditions started with 3% Solvent B for 5 min to wash the sample, followed by a gradient to 40% Solvent B over 40 min, 70% Solvent B at 50 min, 90% Solvent B at 52 min, and held at 90% Solvent B until 60 min. A Waters CapLC system (Waters Corporation, Milford, MA, USA) was used with a flow rate estimated to be 300 nL/min. The mass spectrometer used for electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was a Quadrupole Time-of-Flight (Q-TOF) Global Ultima system (Micromass, Ltd, Manchester, UK) operated with a spray voltage of 3.5 kV. Data-dependent MS/MS was used with a 0.5 s survey scan and 2.5 s MS/MS scans on the three most abundant peaks in the MS survey scan.

Protocol (B) Proteomics Facility, Fred Hutchinson Cancer Research Center— Prior to proteolytic in-gel digestion, the stain from silver-stained gel slices was removed by mixing equal volumes of 30 mM potassium ferricyanide [$K_3Fe(CN)_6$] with 100 mM sodium thiosulfate ($Na_2S_2O_3$) and adding a volume of this mixture to cover the gel slices. The de-staining solution was removed after approximately 5 min and the gel slices were washed repeatedly with 100 mM ammonium bicarbonate (NH_4HCO_3) until they were clear. Gel slice digestions were then performed as described in Shevchenko *et al*. (1996). Following digestion, samples were de-salted using a microC18 ZipTip (Millipore, Bedford, MA), dried, and resuspended in 7 μL of 0.1% TFA. The mass spectrometer used for LC/ESI MS/MS was a LCQ DECA XP mass spectrometer (ThermoElectron, San Jose, CA, USA) as described in Gatlin *et al*. (1998). Data were collected in a data-dependent mode in which an MS scan was followed by MS/MS scans of the three most abundant ions from the preceding MS scan.

Identification of proteins using database search

Mass spectrometry data were searched against a rat protein database (a subset of proteins from the NCBI non-redundant protein database) using the software search algorithms MASCOT (Matrix Science Ltd, London, UK), SEQUEST (ThermoElectron) or COMET (Institute for Systems Biology, Seattle, WA, USA). These searches resulted in the identification of both known and novel mGluR5-interacting proteins.

Files appropriate for MASCOT (pkl files) were created using Masslynx software (Waters, Milford, MA, USA) with a function that smoothes, calculates centroids and assesses the quality of data. MASCOT calculates one statistical parameter to validate protein identification, the Mowse Score. This score is based on the probability (p) that a peptide identified from the experimental fragment matches a peptide in a protein database, and is calculated as: Mowse score = $-10 \times \log(p)$. Thus, a random match will have a high probability value and low Mowse score, while a valid match will have a low probability value and a high Mowse score. MASCOT then ranks the quality of the peptide matches and sums the scores of acceptable peptides to calculate a total protein score. The default total protein score is > 25. Thus, all proteins with a MASCOT total protein score > 25 were considered as real, high confidence (**) proteins that were identified in the mGluR5-signaling complex.

Peptide identification results from the SEQUEST (Eng *et al*. 1994) algorithm were filtered using three result parameters: cross-correlation score (Xcorr), delta correlation value (ΔCn) and ion percentage (%ions). Peptides that were identified with a Xcorr > 2, ΔCn > 0.1 and %ions > 30 were considered to be high confidence peptides. Regardless of whether a single high confidence peptide or multiple high confidence peptides identified a protein, that protein was considered to be a real, high confidence (**) protein, identified in the mGluR5-signaling complex.

A recently developed computer algorithm called COMET was also used for protein identification. The filtering criteria utilized the score, Z-score, dN and ion% parameters from COMET. The score is the dot product between an experimental spectrum and a theoretical spectrum, with the resulting score scaled to 1000. The Z-score is the number of standard deviations away from the mean for the top scoring peptide compared with the top 500 clustered peptide scores. The difference between the normalized scores is represented by the dN parameter, and ion% is the percentage of matched fragment ions over the total number of expected fragment ions for the best matching peptide. Peptides in which + 1 ions had scores greater than 200, + 2 ions had scores greater than 300 and + 3 ions had scores greater than 300, and each of these ions had Z-scores greater than 4.0, dN greater than 0.1 and ion% greater than 30, were considered as real, high confidence peptides. Proteins that were identified with high confidence peptides were considered to be high confidence proteins in the mGluR5-signaling complex.

In this paper, we present 10 proteins that were identified in multiple experiments with multiple high confidence peptides as novel, high confidence (**) molecular constituents of mGluR5-protein complexes (Table 2). In addition, we present proteins that were identified in only one experiment and by at least one high confidence peptide (Table 3). We also include several proteins that were identified by multiple peptides that did not meet the filtering criteria and are identified as low confidence (*) proteins in Tables 1 and 3. The proteins that are low confidence may represent low abundance proteins that result in tandem mass spectra of poorer quality. However, they are included here because of their potential biological relevance as mGluR5-signaling partners based on literature support for either a direct or indirect interaction.

Protein–Protein Interaction Database analysis of mGluR5-interacting proteins

The Protein–Protein Interaction Database (PPID; http://www.anc.ed.ac.uk/mscs/PPID) is an evolving database that arose from the need to interpret proteomic datasets such as those generated by analyzing the NMDA-receptor complex (Husi *et al*. 2000). The PPID includes protein sequence and protein-binding information, together with literature references, that can be queried by online browsing, batch-submission, or by using software like MASCOT. For these studies, the PPID was queried online to determine the degree of separation of the mGluR5 (PPID #A0327) and putative interacting proteins identified in this study. Where a PPID number exists for any given protein, the database provides useful information concerning the likelihood of protein interactions and their linkages based on previously reported data.

Results and Discussion

Immunoprecipitation of mGluR5 from rat brain lysate

The mass spectrometry data depend on the successful immunoprecipitation of mGluR5 from the rat brain lysate under these experimental conditions. Thus, mGluR5 and the closely related mGluR1a were immunoprecipitated from rat brain lysate using subtype-selective antibodies. Parallel experiments were also performed using the negative control anti-CB antibody. The immunoprecipitated proteins from each sample were separated by their molecular mass using 1D gel electrophoresis and transferred to PVDF membrane; the resultant immunoblots were then probed with an mGluR5-selective polyclonal antibody (anti-mGluR5). Figure 1(a) shows that mGluR5 is detected in the total rat brain lysate and the mGluR5 immunoprecipitate, but

is not detected in the CB immunoprecipitate. In addition, Fig. 1(b) shows that mGluR5 is detected in the total rat brain lysate and the mGluR5 immunoprecipitate, but is not detected in the mGluR1a immunoprecipitate. These data show that mGluR5 is specifically immunoprecipitated from rat brain lysates under these experimental conditions.

Mass spectrometry identification of mGluR5 protein

The mass spectrometry results were analyzed for the presence of mGluR5 to prove that the protein is detected by the mass spectrometry, and Fig. 2 shows the LC/MS/MS spectrum that identified mGluR5. Figure 2(a) shows the total ion chromatogram for the proteins that co-immunoprecipitated with mGluR5, Fig. 2(b) shows the mass spectrum of the sample at 25.56 min retention time, and Fig. 2(c) shows the fragmentation mass spectrum of the peak at 782.1 from Fig. 2(b). The software search algorithm SEQUEST identified a total of four peptides at high confidence levels that correspond to peptide sequences in the amino terminal domain of mGluR5, as depicted in Fig. 2(d) (1: DSLISSEEEEGLVR, 2: GLAGEFLLLGSDGWADR, 3: EAVGGITIK and 4: GEVSCCWTCTPCKENEYVFDEYTCK). These data show that mGluR5 is immunoprecipitated from the rat brain lysates under our experimental conditions, and that mGluR5 peptides are identified by LC/MS/MS with high confidence.

Silver stain analysis of proteins that co-immunoprecipitate with mGluR5 and Cascade Blue separated by one-dimensional gel electrophoresis

The proteins that co-immunoprecipitated with mGluR5 were visually compared with those isolated by the negative control, anti-CB antibody. Figure 3 shows that the most abundant protein bands detected in both the mGluR5 and CB immunoprecipitates are the (A) heavy (50 kDa) and the (B) light (25 kDa) immunoglobulin chains (arrows). However, several protein bands that are unique to the mGluR5 immunoprecipitate (Fig. 3, mGluR5) are not detected by the naked eye in the CB immunoprecipitate (Fig. 3, CB). These data show that the mGluR5 sample contains several protein bands that are not detected in the CB negative control, and suggest that these proteins are specifically isolated as molecular constituents of the mGluR5-signaling complex.

Identification of known, high confidence and low confidence molecular constituents of mGluR5-protein complexes

The proteomic approach identified several proteins in the mGluR5 immunoprecipitated samples that are known to directly interact with mGluR5, or that are mGluR5-signaling intermediates, and were not identified in the CB negative control samples (Table 1). Many of these proteins were identified as high confidence (**) proteins based on mass spectrometry filtering criteria. However, several were identified as low confidence (*) proteins (see Table 1). Previous studies support an interaction between each of these proteins and mGluR5. For example, there is a direct interaction between Group I mGluRs and Gq alpha (Abe *et al*. 1992), Go alpha (McCool *et al*. 1998), calmodulin (Minakami *et al*. 1997) and Homer proteins (Brakeman *et al*. 1997;Tu *et al*. 1998). Homer proteins link Group I mGluRs to the inositol trisphosphate receptor (IP3R) (Tu *et al*. 1998;Kammermeier *et al*. 2000), regulate trafficking of mGluR5 (Roche *et al*. 1999) and provide a link between mGluR5 and Shank (Ehlers 1999;Tu *et al*. 1999). Interestingly, the interaction between mGluR5 and Homer, and mGluR5 and Gq/Go, was only identified with confidence in the tolerant rat brain lysate, suggesting that receptor activation leads to altered protein interactions. Finally, previous data support an indirect interaction between mGluR5 and phospholipase C (PLC) beta (Kim *et al*. 1997). Thus, the proteins identified by a proteomic approach are consistent with previous protein interaction studies and PPID queries.

Identification of novel, high confidence molecular constituents of mGluR5-protein complexes in multiple experiments

In addition to known mGluR5-interacting proteins, this proteomic approach also identified 10 mGluR5-interacting proteins in several experiments, at one or both proteomic facilities (Table 2), in both normal and tolerant rat brain lysate. These proteins are considered to be valid, high confidence (**) molecular constituents of the mGluR5-signaling complex, whether direct or indirect, because the identifying peptides met the mass spectrometry filtering criteria for high confidence and were not found in the CB negative control sample. Thus, based on the mass spectrometry results that suggest these novel proteins are valid mGluR5-interactings proteins, we investigated two proteins further to confirm their interaction with mGluR5.

Confirmation of novel, high confidence mGluR5–protein interactions by immunoblot

The identification of mGluR5-interacting proteins is the result of mass spectrometry-identified peptide fragments searched against a rat protein database using the software search algorithms to correlate experimental peptide fragments with theoretical peptide fragments. To validate that the peptide fragments correlated with the identified high confidence interacting proteins, we used immunoblots to specifically test for the presence of these proteins. Figure 4 shows individual immunoblots that contain total rat brain lysate (50 μg protein), mGluR5 immunoprecipitated samples, or CB immunoprecipitated samples probed with antibodies to (a) PACS1 or (b) MAP2. The results show that these high confidence mGluR5-interacting proteins are detected in the total rat brain lysate and the mGluR5 immunoprecipitate, but are not detected in the CB sample, consistent with the mass spectrometry data. Literature searches support potential interactions between mGluR5 and each of these proteins. For example, Group I mGluRs are down-regulated after agonist treatment, possibly due to endocytosis by clathrin-coated pits (Luis Albasanz *et al*. 2002), and in the substantia nigra, more than 80% of immunoreactivity for mGluR5 is intracellular while the majority of mGluR1a is located on the plasma membrane (Hubert *et al*. 2001). This distinct subcellular localization of each Group I mGluR suggests that trafficking and internalization may contribute to the functional role of these receptors in neurons (Lujan *et al*. 1997). A major role for PACS1 is to facilitate the trafficking of proteins between the plasma membrane and the cytosol, and PACS1 is required to maintain phosphorylated furin molecules in a cycling loop between early endosomes and the plasma membrane (Molloy *et al*. 1998). In addition, PACS1 facilitates the movement of furin and the mannose-6-phosphate receptor from the endosome to the Golgi by joining cytoplasmic domain acidic amino acid clusters of the proteins to the adaptor-protein complex-1 (AP-1) in endosomal clathrin-coated membrane pits (Wan *et al*. 1998;Crump *et al*. 2001;Scott *et al*. 2003). As the intracellular carboxy terminal domain of mGluR5 contains several acidic amino acid clusters ($_{969}$EAEE$_{972}$ and $_{1000}$DDD$_{1002}$) and phosphorylation sites (see Hermans and Challiss 2001), PACS1 may play a role in the trafficking of mGluR5 between the plasma membrane and the cytosol.

There is also support for an interaction between mGluR5 and MAP2, a postsynaptic, neuron-specific, microtubule-associated protein that can bind to both microtubules and F-actin, and plays a key role in specific cytoskeletal rearrangements that control the transition from an undifferentiated state to neurite-bearing morphology (for review see Dehmelt and Halpain 2004). MAP2 is found mainly in dendritic shafts (Kaech *et al*. 1997) where it may facilitate transport of membrane-bound receptors, such as NMDA receptors or mGluR5, to the postsynaptic density. Interestingly, glutamate can produce a biphasic change in MAP2 whereby a rapid, transient increase in phosphorylation is mediated by Group I mGluRs and a persistent dephosphorylation of MAP2 is mediated by NMDA receptors (Quinlan and Halpain 1996). The opposing actions of glutamate on the phosphorylation state of MAP2 regulate its interaction with microtubules and actin filaments, and suggests that glutamatergic regulation

of MAP2 phosphorylation may transduce neural activity into modifications in dendritic structure.

Identification of novel, high confidence and low confidence molecular constituents of mGluR5-protein complexes in single experiments

This proteomic approach identified many novel high confidence (**) mGluR5-interacting proteins in a single experiment that were not detected in the CB negative control sample, and approximately 50% of these identifications came from the tolerant rat brain lysate. We also included several novel low confidence (*) mGluR5-interacting proteins identified in several experiments that were not detected in the CB negative control sample. Inclusion of the low confidence proteins is supported by data in Table 1, which lists several known mGluR5-interacting proteins that are designated as low confidence proteins based on the filtering criteria, but are known to be valid mGluR5-interacting proteins. In addition, prior studies support a potential role for many of the proteins in Table 3 as mGluR5-interacting proteins, and they may represent valid signaling intermediates or effectors in the mGluR5-signaling complex. For example, Citron and Group I mGluRs are both expressed in distinct hippocampal interneuron populations (Zhang *et al*. 1999;van Hooft *et al*. 2000) where Citron may contribute to the regulation of Group I mGluR-mediated release of GABA from these cells. In addition, Bassoon, a 420 kDa protein localized at the active zone of presynaptic nerve terminals that is involved in cytomatrix organization at the neurotransmitter release site (tom Dieck *et al*. 1998), and electron microscopy reveals mGluR5 immunoreactivity on presynaptic axon terminals (Romano *et al*. 1995). Physiology studies suggest that a presynaptic mGluR is responsible for the induction of long-term depression (LTD) in hippocampal CA1 (Faas *et al*. 2002) and interestingly, this mGluR-induced LTD can be completely blocked by (2-methyl-6-(phenylethynyl)-pyridine (MPEP), a specific mGluR5 antagonist. Thus, mGluR5 regulation of presynaptic neurotransmitter release from hippocampal synapses may involve mGluR5 protein interactions with Bassoon.

Confirmation of a novel, high confidence mGluR5–protein interaction, identified in a single experiment, by immunoblot

To validate that these high confidence peptide fragments identified in a single experiment correlated with the identified protein and interacted with mGluR5, we used immunoblot to specifically analyze the protein interaction. Figure 5 shows an immunoblot that contains total rat brain lysate (50 μg protein), mGluR5 immunoprecipitated sample, or CB immunoprecipitated sample probed with an antibody to Dynamin1. The results show that this mGluR5-interacting protein, identified in only one experiment, is detected in the total rat brain lysate and the mGluR5 immunoprecipitate, but is not detected in the CB sample, consistent with the mass spectrometry data. There is support for the interaction of mGluR5 and Dynamins. Dynamins interact with the Src homology 3 (SH3) domain of PLCγ (Seedorf *et al*. 1994;Okamoto *et al*. 1997), and the pleckstrin homology (PH) domain of Dynamin binds phosphatidylinositol-4,5-bisphosphate (PIP2) (Zheng *et al*. 1996). Both PIP2 and G protein βγ subunits can regulate Dynamin I GTPase activity (Lin and Gilman 1996), although sequestering Gβγ subunits inhibits clathrin-coated endocytosis (Lin *et al*. 1998). Recently, Dynamin was shown to be necessary for endocytosis (for review see Sever 2002) and Dynamin 2 contributes to the clathrin-independent endocytosis of mGluR5 in COS-7 cells (Fourgeaud *et al*. 2003). In addition, Dynamin 3 is found in complex with Homer and mGluR5 (Gray *et al*. 2003), and there is a direct interaction between Dynamin 2 and Shank (Okamoto *et al*. 2001) and members of the Shank/ProSAP family of post-synaptic density scaffolding proteins. These data are consistent with a role for Dynamins in glutamate receptor down-regulation, and support the interaction between mGluR5 and Dynamin in our proteomic studies.

Confirmation of mGluR5–protein interactions by reverse immunoblot

To strengthen the existing data for an interaction between mGluR5 and Dynamin, MAP2 and PACS1, we used reverse co-immunoprecipitation of each reported protein and probed for mGluR5 immunoreactivity. Figure 6 shows a reverse immunoblot that contains total rat brain lysate (50 μg protein) and Dynamin1, MAP2 or PACS1 immunoprecipitated samples, as well as the CB immunoprecipitated sample probed with an antibody to mGluR5. The results show that a weak band was detected in the Dynamin 1 immunoprecipitate, and a stronger band was detected in the MAP2 immunoprecipitate, both at molecular weights that correspond to the dimer form of mGluR5. In contrast, there is a very strong band in the PACS1 immunoprecipitate at the molecular weight that corresponds to the monomer form of mGluR5. These findings are consistent with the mass spectrometry data. Dynamin was detected in the proteomic analysis of mGluR5 co-immunoprecipitates from ischemic pre-conditioned rat brain, representing activated receptor. MAP2 was detected in several experiments, and PACS1 was abundantly detected in virtually every co-immunoprecipitation that we analyzed. Thus, we feel that these data support true interaction between these novel proteins and mGluR5 in rat brain.

Identification of proteins detected in both mGluR5 and CB samples

This proteomic approach identified several proteins as mGluR5-interacting proteins at high confidence levels, but these proteins were also identified in the CB negative control samples. Most of these proteins contribute to the organization of subcellular structures and are involved in membrane skeleton organization. They include several isoforms of actin, actinin, ankyrin, cofilin, dynein, fibrillin, myosin, plecktin, spectrin, tubulin and vimentin, and their identification is consistent with previous studies that did not analyze a stringent negative control by mass spectrometry; their identification in the negative control may reflect their high abundance in the sample. For example, actin, actinin, spectrin and myosin were all reported as protein components of the NMDA receptor multiprotein complex (Husi *et al*. 2000). In addition, previous studies support interactions between cytoskeletal proteins and mGluRs. Actin binds to Cupidin (Homer 2a) that in turn binds to mGluR1a (Shiraishi *et al*. 1999), and actin polymerization/depolymerization regulates the movement of mGluR5 in the plasma membrane (Sérge *et al*. 2003). Actin also regulates the release of calcium from the endoplasmic reticulum in cultured hippocampal neurons (Wang *et al*. 2002), and actin-dependent changes in dendritic spines may play a role in neuronal plasticity (Matus and Shepherd 2000). Furthermore, studies show that mGluR5 binds to microtubules (Sérge *et al*. 2003), mGluR1a interacts with β-tubulin (Ciruela *et al*. 1999) and mGluR7a interacts with α-tubulin (Saugstad *et al*. 2002). Similarly, the muscarinic receptor agonist, carbachol, induces a rapid and transient translocation of tubulin to the plasma membrane, microtubule reorganization and a change in cell shape (Popova 2000). Thus, microtubules play a role in G protein-coupled receptor targeting and organization during synapse formation, and G protein-coupled receptors modulate cytoskeletal dynamics, intracellular trafficking and cellular architecture; the detection of cytoskeletal proteins in the CB negative control sample may simply reflect the high abundance of these proteins in the samples.

In conclusion, we utilized a proteomic approach to identify novel proteins that may regulate mGluR5 responses by direct or indirect protein interactions. This approach does not rely on the heterologous expression of proteins and offers the advantage of identifying protein interactions in a native environment. Thus, mGluR5 was immunoprecipitated from rat brain lysates, and the co-immunoprecipitating proteins were originally removed *en masse* from the protein A sepharose beads and subjected to liquid chromatography and mass spectrometry. However, this method sometimes resulted in a sample complexity that made the mass spectrometry analysis difficult and thus, the sample preparation procedure was modified to include separation of the protein samples by 1D gel electrophoresis; this is the preferred method for future studies. The peptide sequences identified by mass spectrometry were then matched

to protein databases to identify correlating parent proteins. This proteomic approach revealed the interaction of mGluR5 with known regulatory proteins, as well as novel interacting proteins that may reflect previously unidentified molecular constituents of the mGluR5-signaling complex. As immunoblot analysis and reverse co-immunoprecipitation confirmed the interaction between high confidence mGluR5-interacting proteins, such as PACS1 and MAP2, and Dynamin, this approach has successfully led to the identification of novel proteins that may be molecular constituents in the mGluR5-signaling complex. Interestingly, the reverse immunoblots showed that Dynamin1 and MAP2 interact with the dimer form of mGluR5, while PACS1 interacts with the monomer form of mGluR5. These data are intriguing and may represent protein interactions at distinct subcellular regions. For example, immunogold studies revealed that in rat substantia nigra, mGluR1a immunoreactivity was primarily detected at the plasma membrane, while > 80% of mGluR5 immunoreactivity was intracellular (Hubert *et al*. 2001). As a major role for PACS1 is to facilitate the trafficking of proteins between the plasma membrane and the cytosol, the interaction of monomer mGluR5 with PACS may reflect sequestering of mGluR5 in the intracellular compartment. Alternatively, while little is known about the functional role for mGluR dimers (see Romano *et al*. 2001), it is possible that the interaction of Dynamin1 and MAP2 with the dimer form of mGluR5 may reflect protein interactions at the plasma membrane, based loosely on evidence that the extracellular ligand binding domain of mGluR1 can form homodimers (Kunishima *et al*. 2000).

In addition, PPID searches support a link between mGluR5 and several proteins identified in this study, and several of these proteins are now the focus of new studies in our laboratory to determine their role in mGluR5-signaling in the CNS. These data support the use of a proteomic approach to identify novel protein interactions in native tissue, which will be particularly useful for neurobiology applications where distinct protein interactions can dramatically alter the outcome of the response to neurotransmitter release, or the disruption of normal protein interactions can lead to severe neurological and psychiatric disorders. For example, there is evidence that a genetic defect in DISC1 (disrupted in schizophrenia 1) leads to the disruption of a trimolecular protein complex that may in part lead to the developmental onset of schizophrenia (Brandon *et al*. 2004). In addition, studies show that a physical interaction between NMDA receptors and dopamine receptors leads to increased plasma membrane insertion of dopamine receptors (Pei *et al*. 2004). Thus, disruption of NMDA receptor-mediated up-regulation of dopamine receptor function may serve an as underlying molecular mechanism that leads to the onset of schizophrenia. New opportunities now exist for the study of complex protein interactions due to significant advancements in mass spectrometry and protein identification provided by large-scale nucleotide sequencing, and these approaches can be used to study the molecular constituents of signaling complexes and the alterations in protein interaction that may underlie neuropsychiatric or neuropathological disorders.

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Abbreviations used

ACN
: acetonitrile

AMPA
: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

CB
Cascade Blue

CTD
carboxy terminal domain

ESI
electrospray ionization

FA
formic acid

GluRs
glutamate receptors

GST
glutathione-*S*-transferase

HRP
horseradish peroxidase

IP3R
inositol triphosphate receptor

LC
liquid chromatography

MAP2
microtubule-associated protein 2

mGluR5
metabotropic glutamate receptor 5

MS
mass spectrometry

MS/MS
tandem mass spectrometry

NMDA
N-methyl-D-aspartate

PACS1a
phosphofurin acidic cluster sorting protein 1

PAS
protein A-sepharose

PKC
protein kinase C

PLC
phospholipase C

PPID
Protein–Protein Interaction Database

PSD
postsynaptic density

PVDF
polyvinylidene difluoride

Q-TOF
Quadrupole Time-of-Flight

TBS
Tris-buffered saline

TFA
trifluoroacetic acid

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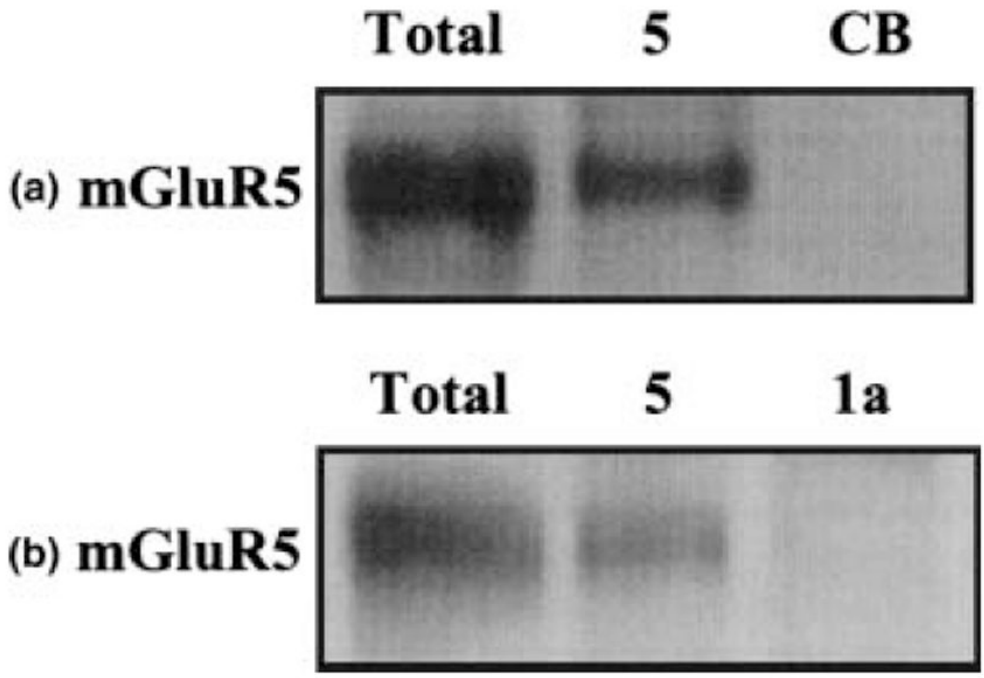

Fig. 1.

Immunoprecipitation of mGluR5 from rat brain lysate. (a) Total rat brain lysate (Total), mGluR5 (5) and Cascade Blue (CB) immunoprecipitates probed with anti-mGluR5. (b) Total rat brain lysate (Total), mGluR5 (5) and mGluR1a (1a) immunoprecipitates probed with anti-mGluR5.

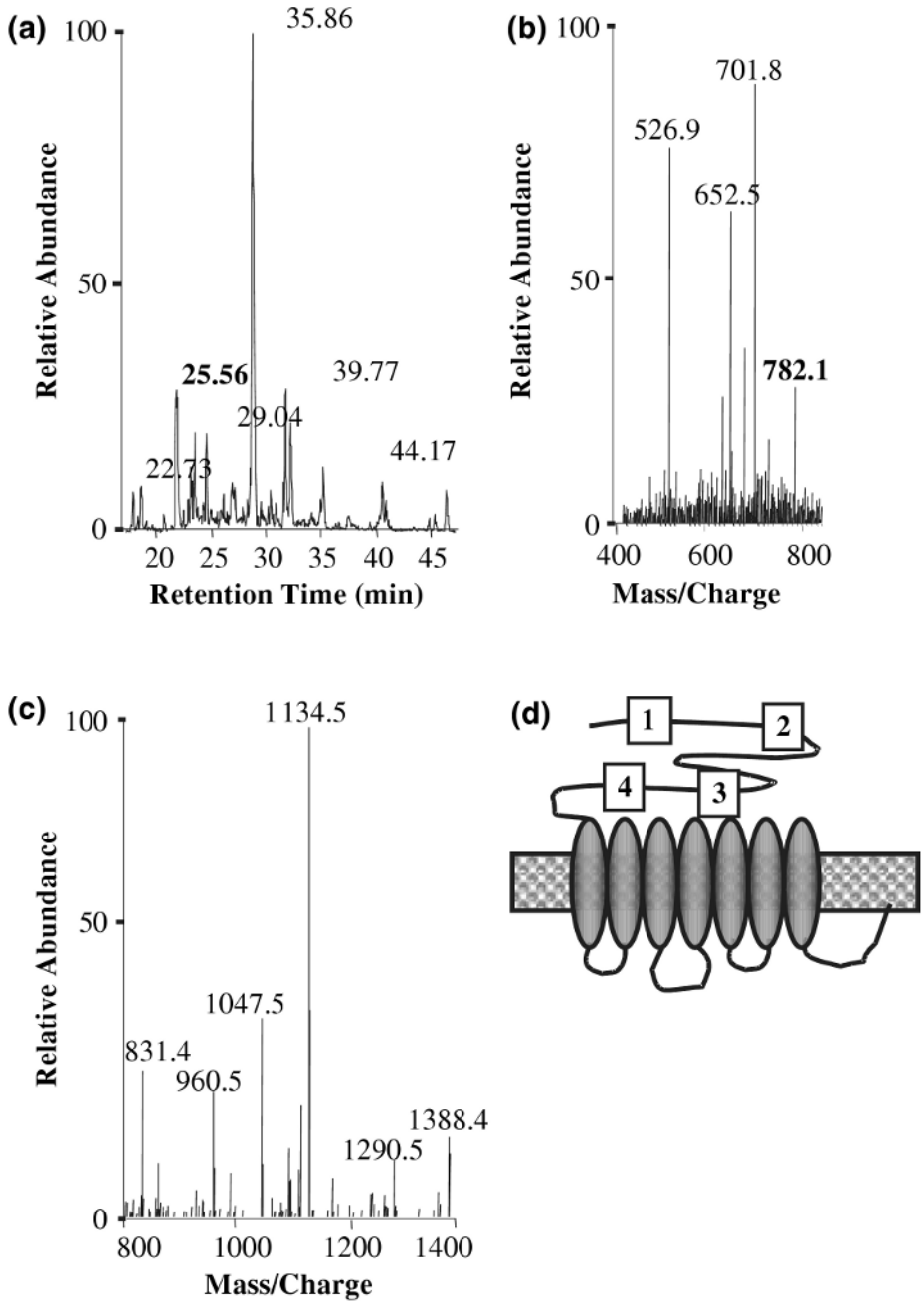

Fig. 2.

Identification of mGluR5 peptides by mass spectrometry. (a) Total ion chromatogram for the mGluR5 sample. (b) Mass spectrum at 25.56 min retention time. (c) Tandem mass spectrum of m/z 782.1 from Fig. 2(b). (d) Diagramatic representation of peptides identified in the second mass spectrometry.

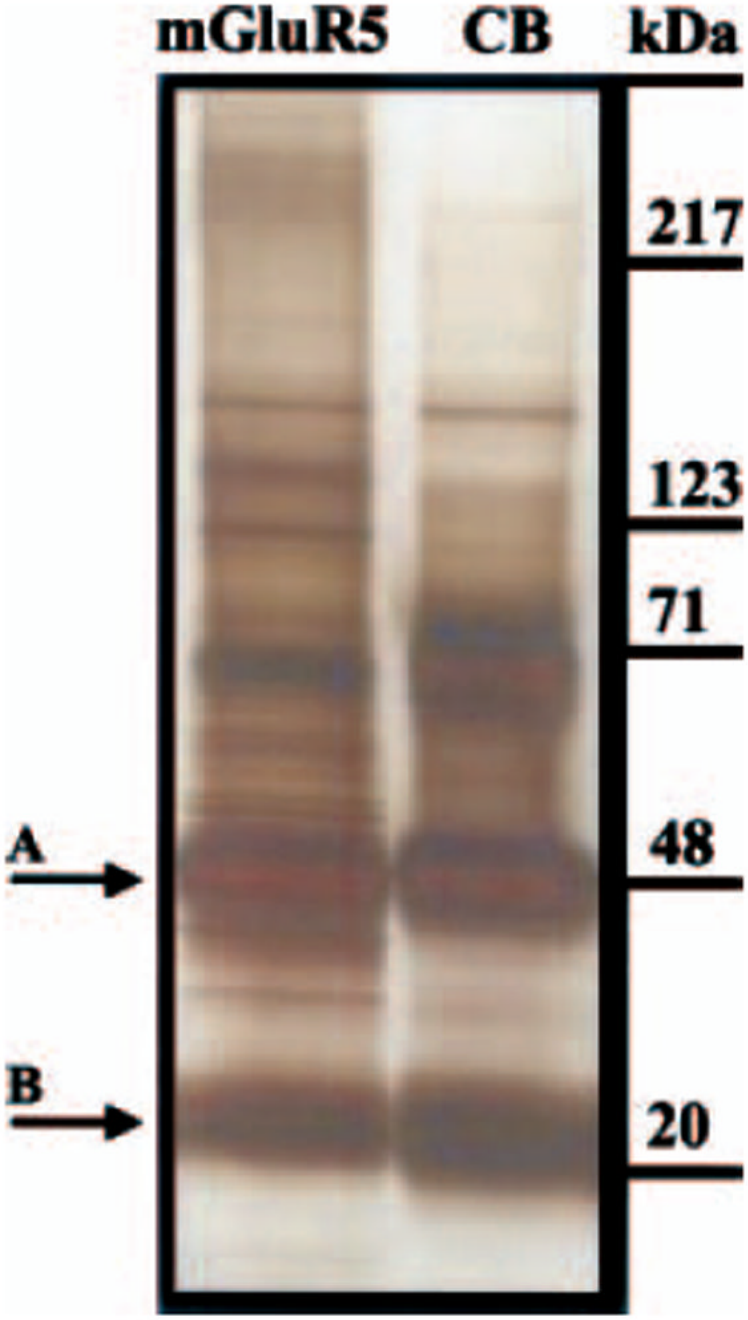

Fig. 3.

Silver stain analysis of proteins that co-immunoprecipitate with mGluR5 and Cascade Blue separated by one-dimensional gel electrophoresis. Arrows indicate (A) heavy and (B) light chain immunoglobulin subunits of the primary antibodies.

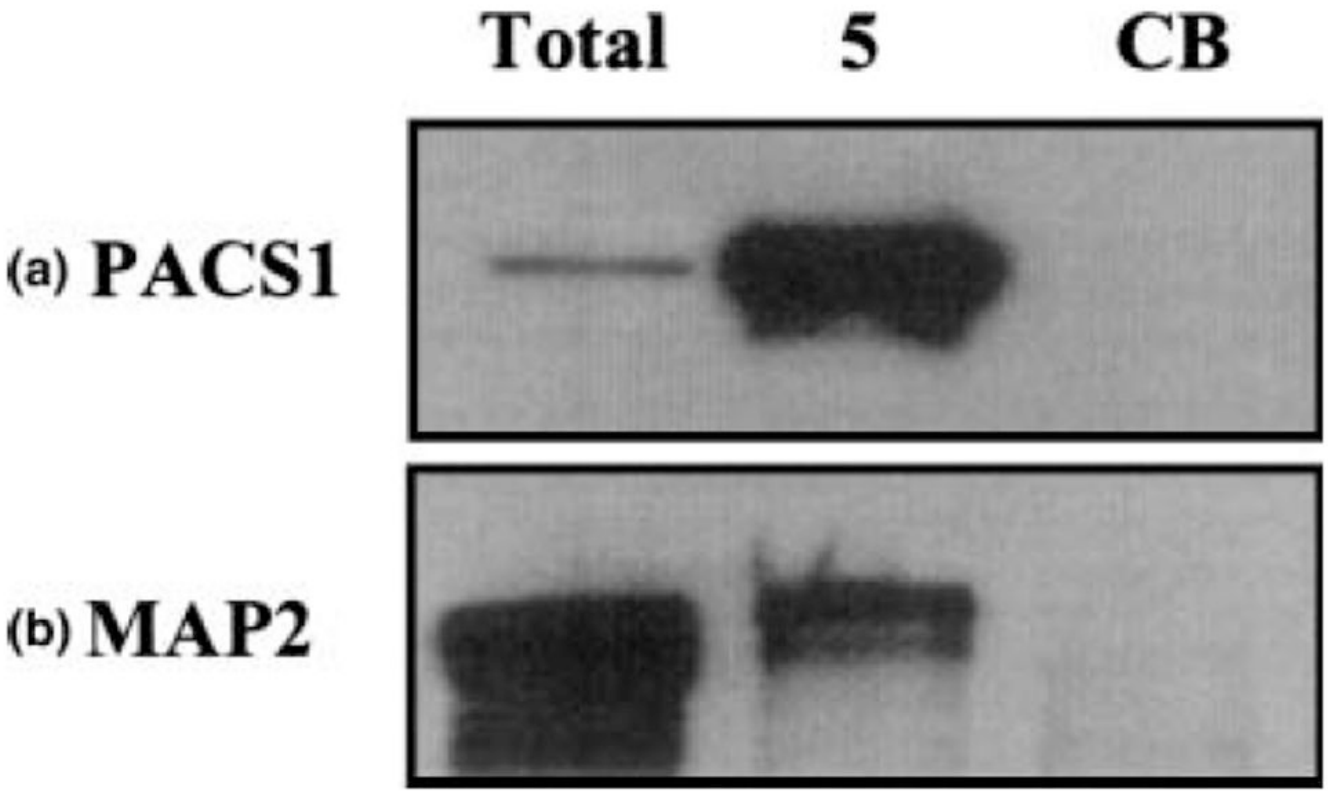

Fig. 4.
Confirmation of novel, high confidence mGluR5–protein interactions by immunoblot. Total rat brain lysate (Total), mGluR5 (5) and CB (CB) immunoprecipitates probed with (a) anti-PACS1 and (b) anti-MAP2.

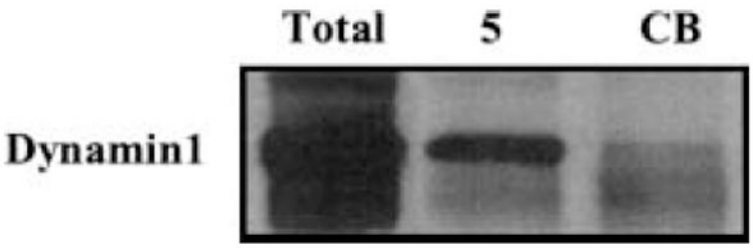

Fig. 5.

Confirmation of a novel, high confidence mGluR5–protein interaction, identified in a single experiment, by immunoblot. Total rat brain lysate (Total), mGluR5 (5) and CB (CB) immunoprecipitates probed with anti-Dynamin1.

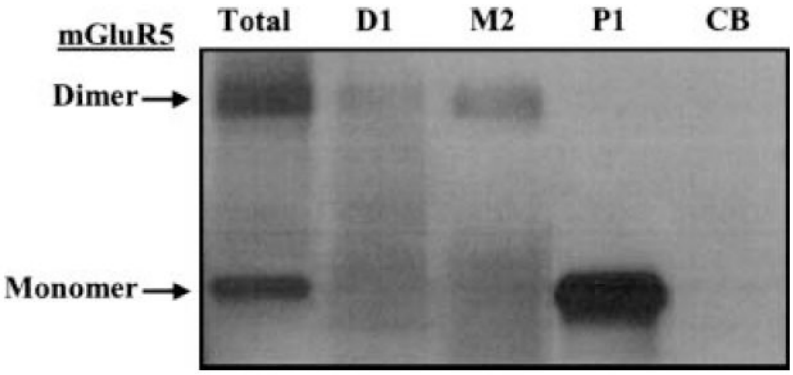

Fig. 6.

Confirmation of mGluR5–protein interactions by reverse immunoblot. Total rat brain lysate (Total), or Dynamin1 (D1), MAP2 (M2), PACS1 (P1) and CB (CB) immunoprecipitates probed with anti-mGluR5.

Table 1

Known high and low confidence molecular constituents of mGluR5-protein complexes

Name	Rattus Gi number	Protocol	PPID (Separation)
Homer 3**	16758964	B##	A0104 (1)
GTP-binding protein alpha q (Gq)**	9296968	B##	A0041 (1)
GTP-binding protein alpha o (Go)**	8394152	B##	A0041 (1)
Calmodulin**	1334203	A,B##	A0008 (3)
Inositol 1,4,5-triphosphate receptor 1 (IP3R1)**	111838	A,B#,##	A0154 (2)
Inositol 1,4,5-triphosphate receptor 2 (IP3R2) *	111839	A#	No PPID
Phospholipase C-beta-1 (PLC-beta-1)*	130223	B#	A0330 (2)
Shank 1a*	13929054	A#	A0075 (2)

** Identified by at least one high confidence peptide.

* Identified by multiple peptides that did not meet the filtering criteria.

En masse off beads,

1D gel.

Table 2

Novel, high confidence molecular constituents of mGluR5-protein complexes, identified in multiple experiments

Name	Rattus Gi number	Protocol	PPID (Separation)
14-3-3 gamma**	9507245	A,B##	A0362 (4)
14-3-3 theta**	6981712	B##	A0907 (5)
14-3-3 zeta**	13487931	A, B##	A0361 (3)
Adaptor-related protein complex (AP2), beta subunit**	18034787	A,B#,##	A0253 (4)
Cell division cycle 10 (CDC10), rat homolog**	12018296	A,B#,##	No PPID
CDCrel-1a**	8953677	B#,##	No PPID
Microtubule-associated protein 1a (MAP1a)**	13591886	B#,##	A0085 (3)
Microtubule-associated protein 2 (MAP2)**	547890	A,B ##	A0134 (3)
Phosphofurin acidic cluster sorting protein 1a (PACS1)**	19705535	A,B##	No PPID
Phosphofurin acidic cluster sorting protein 1b (PACS1b)**	3347955	B##	No PPID

** Identified by at least one high confidence peptide.

En masse off beads,

1D gel.

Table 3

Novel, high and low confidence molecular constituents of mGluR5-protein complexes, identified in single experiments

Name	Rattus Gi number	Protocol	PPID (Separation)
14-3-3 beta**	9507243	A##	A0467 (4)
14-3-3 eta**	6981710	B##	A0633 (3)
Adducin, alpha**	1083589	B##	No PPID
Adducin, beta**	10720378	B##	A0587 (4)
Aggrecan 1*	11990616	A#	No PPID
Aldolase A**	6978487	B##	A1923 (ND)
Aldolase C**	6978489	B##	A0428 (3)
Bassoon*	9506427	A#	A0285 (ND)
Calretinin**	16758892	B##	A0437 (ND)
Casein kinase II, beta**	13591930	B##	A0753 (4)
Clathrin heavy chain**	9506497	B##	A0119 (4)
Citron*	2745840	A#	A0276 (4)
Destrin**	7441446	B##	No PPID
Dynamin 1**	18093102	B##	A0095 (3)
Dynamin 2**	1083647	B##	A1927 (ND)
Endophilin 1**	10720268	B##	A0256 (3)
Fibronectin**	9506703	A#	A1466 (3)
Glutamate receptor interacting protein (GRIP)*	14091754	A#	A0301 (5)
Histamine receptor H1**	8393564	B##	No PPID
Myristoylated alanine-rich C-kinase substrate (MARCKS)**	266495	B##	No PPID
Microtubule-associated protein 1b (MAP1b)**	1083716	B##	A0625 (3)
Mitogen-activated protein kinase 12*	3023701	A#	A0293 (4)
NBC (Na/HCO3)-like protein*	9437326	B##	No PPID
N-ethylmaleimide sensitive factor (NSF)*	13489067	B##	No PPID
Na^+,K ± ATPase alpha 1**	6978543	B##	A0340 (4)
Na^+,K ± ATPase alpha 2**	6978545	B##	A0341 (ND)
Na^+,K ± ATPase alpha 3**	19855078	B##	A0342 (ND)
Na^+,K ± ATPase beta 1**	6978549	B##	A0344 (ND)
Na^+,K ± ATPase beta 2**	6978551	B##	A0345 (ND)
N-methyl D-aspartate (NMDA) receptor 2a*	31377498	A#	A0002 (3)
Neural cell adhesion molecule L1 (N-CAM L1)**	13928706	B##	A0290 (4)
Nuclear factor regulated by interleukin 3**	16758542	A#	No PPID
Osteopontin**	6981580	A#	A1526 (5)
Periaxin*	18677712	A#	No PPID
Phosphatidylethanolamine binding protein**	406294	B##	A1328 (5)
Phospholipase C-delta	130228	A#	No PPID(PLC-delta)*
Phospholipase C-epsilon	16758594	A,B#	No PPID(PLC-epsilon)*

Name	Rattus Gi number	Protocol	PPID (Separation)
Plasma membrane calcium-transporting ATPase (PMCA1)**	14286099	B##	No PPID
Profilin II**	13540707	B##	A0524 (3)
Ras-related small GTP binding protein 3a (RAB3a)**	6981452	B##	A0037 (4)
Ras-related small GTP binding protein 10 (RAB10)**	420269	B##	A0621 (6)
Ras-related small GTP binding protein 27b (RAB27b)**	16758202	B##	A0360 (ND)
RAB GDP dissociation inhibitor**	1707888	B##	A0567 (5)
RABphilin 3a*	19424162	B##	A0212 (4)
Reelin*	31543579	A#	A1119 (5)
SNAP-25-interacting protein (SNIP)*	9507127	A#	No PPID
Solute carrier family 1, member 3**	9507115	B##	No PPID
Solute carrier family 12, member 5**	19705463	B##	No PPID
Synaptogyrin 1**	9507167	B##	A0288 (ND)
Synaptojanin 1**	8134729	B##	A0120 (3)
Synaptopodin**	11067429	B##	No PPID
Utrophin*	6981696	A,B#	A0752 (3)
Vacuolar ATP Synthase F*	1718093	A#	A0405 (6)
Vesicle associated membrane protein (VAMP) 2b**	4894188	B##	No PPID
Voltage-dependent calcium channel, alpha 1a**	6978579	B##	No PPID
Voltage-dependent calcium channel, L-type beta*	16758716	B#	No PPID
Voltage-activated calcium channel, T-type alpha 1*	21687094	A##	No PPID

** Identified by at least one high confident peptide.

* Identified by multiple peptides that did not meet the filtering criteria.

En masse off beads,

1D gel.