The ataxic and epileptic mutant mouse stargazer (stg), arose spontaneously as a consequence of a viral insertion of a 6-kb early transposon in intron 2 of the stargazer gene (1, 2). The mutation results in premature transcriptional arrest and complete ablation of stargazin expression (3, 4). From P14 onwards stg display phenotypic consequences of the mutation that includes head tossing due to an inner ear defect (2), ataxia and impaired conditioned eyelid reflex, both a consequence of cerebellar defects (5) and absence epilepsy (6). The molecular basis for these disparate defects has still to be unequivocally resolved but ultimately these must be direct or downstream consequences of ablated expression of stargazin. Based on low sequence homology to the skeletal muscle-specific L-type voltage-gated calcium channel (VGCC)γ1 subunit, stargazin was proposed to be a brain-specific γ isoform, and in this context it was named CACNG8 (2). Heterologous co-expression studies showed that stargazin had relatively minor effects on P/Q- and α1L T-type VGCC kinetics and cell surface trafficking (2, 7, 8, 9, 10). It has recently been shown that the N-type VGCC α1B subunit co-precipitates with immunoprecipitated stargazin from detergent soluble mouse brain (4). Cerebellar GABA_A receptor expression is also severely compromised in stargazer mice (11, 12). The apparent fickle nature of stargazin associations was compounded when it was shown that it also played an intimate role in the trafficking and synaptic targeting of AMPA receptors to cerebellar granule cell synapses through a VGCC-activity-independent mechanism (13). Heterologous expression studies revealed that stargazin co-associates with AMPAR GluR1, -2, and -4 (but not NMDA receptor subunit, NR1) and PDZ domains of postsynaptic density proteins, e.g. PSD-95 (13, 14). Sharp et al. (4) have since provided evidence that stargazin complexes with GluR1 in vivo, this has been confirmed and expanded upon by Tomita et al. (15) to include GluR2 and GluR4. The stargazin ability to influence recruitment of AMPA receptors to the mossy fiber-cerebellar granule cell synapse is, at least in part, dictated by its C-terminal RRTTPV sequence that confers interaction with PDZ domain-containing proteins such as PSD-95. The extent to which AMPA receptor subunits are targeted/clustered at synapses in a stargazin-mediated manner appears to be limited by the number of available PSD-95 molecules with which stargazin-AMPAR complexes can interact while the availability of stargazin seems to dictate the extent of surface-trafficked AMPA receptors (14). It is not yet known, however how stargazin regulates AMPA receptor trafficking, nor whether other stargazin-protein interactions are required for synaptic targeting or synaptic stability of receptors already docked at the synapse. The work described herein shows that stargazin interacts with light chain 2 (LC2) of microtubule-associated protein 1A (MAP1A) in vivo and that this complex includes GluR2 but not PSD-95, SAP97, nor actin. LC2 is synthesized from MAP1A mRNA, which is translated to give a precursor protein that is subsequently cleaved into the light chain 2 (LC2) of microtubule-associated protein 1A (MAP1A) in vivo and that this complex includes GluR2 but not PSD-95, SAP97, nor actin. LC2 is synthesized from MAP1A mRNA, which is translated to give a precursor protein that is subsequently cleaved into the light chain 2 (LC2) of microtubule-associated protein 1A (MAP1A) in vivo and that this complex includes GluR2 but not PSD-95, SAP97, nor actin. LC2 is synthesized from MAP1A mRNA, which is translated to give a precursor protein that is subsequently cleaved into the light chain 2 (LC2) of microtubule-associated protein 1A (MAP1A) in vivo and that this complex includes GluR2 but not PSD-95, SAP97, nor actin.

The ataxic mutant mouse stargazer is a null mutant for stargazin, a protein involved in the regulation of cell surface trafficking and synaptic targeting of AMPA receptors. The extreme C terminus of stargazin (sequence, −TTPV), confers high affinity for PDZ domain-containing proteins e.g. PSD-95. Interaction with PDZ proteins enables stargazin to fulfill its role as an AMPA receptor synaptic targeting molecule but is not essential for its ability to influence AMPA receptor trafficking to the neuronal cell surface. Using the yeast-two hybrid approach we screened for proteins that interact with the intracellular C-terminal tail of stargazin. Positive interactors included PDZ domain-containing proteins e.g. SAP97, SAP102, and PIST. Interestingly, light chain 2 of microtubule-associated protein 1 (LC2), which does not contain PDZ domains, was also shown in independent work to be a direct interactor that occurred upstream of the −TTPV sequence of stargazin. Immunoprecipitations of Triton X-100 soluble cerebellar extracts revealed that LC2 is pulled down not only by anti-stargazin antibodies but also anti-GluR2 antibodies suggesting that stargazin and AMPA receptor subunits associate with LC2. Immunopurified full-length, native stargazin was shown to co-associate not only with GluR2 in vivo but also with full-length, native LC2. Indeed, LC2 co-associates with stargazin when part of a tripartite complex comprising LC2-stargazin-GluR2. Since this complex was extracted using Triton X-100 and was devoid of PSD95, SAP97, and actin we postulate that LC2 is involved in trafficking of AMPA receptors in cerebellar neurons before they are anchored at the synapse.

The abbreviations used are: VGCC, voltage-gated calcium channel; TM, transmembrane; TARP, transmembrane AMPA receptor regulatory protein; MAP, microtubule-associated protein; GABAR, γ-aminobutyric acid Type A receptors; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
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EXPERIMENTAL PROCEDURES

Animals—Wild-type (C3B6F1; +/+), heterozygous (C3B6F1; +/+stg) and homozygous stargazer mutant mice (C3B6F1; stg/stg) were obtained from heterozygous breeding pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Durham vivarium on a 12 h light/dark cycle with food and water available ad libitum. Animal husbandry, breeding, and procedures performed during the experiments were conducted according to the Scientific Procedures Act 1986. We, in accordance with others (19, 20), have found no differences between wild-type (+/+ and heterozygous (+/+stg) mice in terms of their phenotype, behavior or any of the molecular entities we have studied. We routinely use, therefore, a mixture of +/+ and +/+stg mice in our control experiments. New Zealand White (NZW) rabbits used for polyclonal antibody production were obtained from B & K Universal Ltd. (Hull).

Yeast Two-hybrid Screen—The C-terminal stargazin bait representing amino acid residues 202–232 was constructed by PCR amplification and cloned into pGBK7T (Clontech). DNA was sequenced from clones to confirm the correct orientation and sequence accuracy. A 77 transcrip- tion unit (UTS) followed by translation in a rabbit reticulocyte system (T7, Promega) was performed from the pGBK7T clone, which has a c-Myc tag inserted upstream of the bait. The expected size protein product was detected by immunoblotting, performed as previously described by Thompson and Stephenson (21). The bait plasmid was introduced into yeast strain AH109 by the lithium acetate-mediated method, a protocol provided by Clontech, which was adapted from Gietz et al. (22). The bait was used to screen an adult mouse brain MATCHMAKER cDNA library (Clontech), constructed in pACT2 and pre-transformed into yeast strain Y187, in a mating reaction, which was plated onto S.D. agar deficient in leucine, tryptophan, and histidine (a low stringency media) to confirm the phenotype.

Clone Analysis—DNA was extracted from cultures of each clone by standard methods and then PCR-amplified with pACT2-specific primers (AD-2D Insert Screening Amplimer Set, Clontech). Identical clones were identified by restriction enzyme digest analysis, and hybridization of probes to DNA dot blots (standard methods). To identify PDZ proteins 2125 bp F[BS]-hybridization probe (encompassing all three PDZ domains) were identified by restriction enzyme digest analysis, and hybridization methods. Yeast mating assays were performed using constructs and plasmids transformed into yeast. Identification of stargazin with the anti-stargazin antibody that leached off the streptavidin beads coupled to the immunoaffinity columns, was applied as for the C-terminal stargazin bait representation. The bait plasmid was introduced into yeast strain AH109 by the lithium acetate-mediated method, a protocol provided by Clontech, which was adapted from Gietz et al. (22). The bait was used to screen an adult mouse brain MATCHMAKER cDNA library (Clontech), constructed in pACT2 and pre-transformed into yeast strain Y187, in a mating reaction, which was plated onto S.D. agar deficient in leucine, tryptophan, and histidine (a low stringency media). Colonies grew in 3 days and were picked and re-screened on high stringency media to confirm the phenotype.

Cloning and Expression of Protein Constructs—LC2 residues 2554–2774, stargazin residues 202–323 (stargazin-full), and stargazin residues 202–317 (stargazin-truncated) were cloned into pENTR/D, then recombined into pDEST17 (Invitrogen), which is a N-terminal His6 tag. Constructs were sequenced to confirm their open reading frames, then transformed, and then expressed in Escherichia coli strain BL21-AI. Proteins were purified on Ni-nitrilotriacetic acid columns (Invitrogen), and competent expression was verified by immunoblot analysis.

Gel Overlay Analysis—Essentially as Wu et al. (24) in the following modifications: Proteins (1 μg) were subjected to SDS-PAGE and transferred to nitrocellulose filters. Filters were then initially im- mersed in 6 μg guanidine- HCl for 10 min at 4 °C followed by six sequential 10 min, 4 °C incubations in 1:1 serially diluted guanidine-HCl (final incubation in 0.9575 μl). Filters were blocked in Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with 10% (w/v) fat-free skimmed dry milk and 0.3% (v/v) Tween-20 for 30 min. The block was discarded and the filters rinsed twice with incubation buffer (TBS with 0.25% (w/v) milk, 0.3% (v/v) Tween-20, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) before over- night incubation in the overlay protein at 17–30 μg/ml at 4 °C in TBS containing 4% (v/v) milk. Filters were washed four times with 0.3% (v/v) Tween-20, 2.5% (w/v) milk with the second wash also containing 0.0001% (v/v) glutaraldehyde. The presence of overlaid proteins on the filter was detected by immunoblotting.

SDS-PAGE Gel Silver Staining—Proteins were separated by SDS- PAGE, gels were fixed by 2× immersion for 15 min in fixative (10% (v/v) acetic acid : 40% (v/v) methanol) followed by 30 min in 250 ml of sensitizer (30% methanol, 13 mM sodium thiosulfate, 83 mM sodium acetate). Gels were then washed × 5 min in H2O and then exposed to 15 mm silver nitrate for 20 min. After washing 2× 1-min washes in H2O, gels were exposed to developer (24 mM sodium carbonate, 0.15% (v/v) formaldehyde in 250 ml H2O) until silver-stained protein bands emerged on the gel. Development was terminated by addition of a saturated aqueous solution of EDTA.

Immunofluorescence Purification of Stargazin—Adult mouse cerebella from control (+/+ and +/+stg) and stargazer mice were rapidly dissected and flash frozen in liquid nitrogen and stored at −70 °C until required. Cerebellar membrane homogenates of both sets of tissue were simulta- neously prepared according to Kannelberg et al. (25). Briefly, cerebella were rapidly thawed and homogenized in buffer A comprising 10 mM HEPES, pH 7.1, 100 mM KCl, 2 mM MgCl2, 1 mM EDTA, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors leupeptin (1 μg/ml), aprotinin (2 μg/ml) and pepstatin A (1 μg/ml) all at 4 °C. The homogenates were centrifuged at 45,000 × g, 5 min, 4 °C. The supernatant on buffer A supplemented with 1 mM Triton X-100, stirred for 30 min at 4 °C before ultracentrifugation at 147,000 × g for 75 min, 4 °C. The Triton X-100 soluble material was passed through glass wool before being applied to separate, but identi- cal, anti-stargazin antibody-coupled agarose gels (1.5-ml bed volume, hereafter termed the immunofluorescence columns) in 15-ml conical tubes and incubated with agitation, overnight at 4 °C. The gels were poured into 15-ml Bio-Rad columns. Unbound material was allowed to elute off the column and was collected for analysis (flow-through). The immuno- affinity columns were subsequently washed with 50-column volumes of buffer A supplemented with 1.0% (v/v) Triton X-100. Antibody-bound proteins were eluted from the respective columns by one of the following methods: eluting the primary F (5′-TCTG-CTCTGTTAGTAGACAAACA-3′) and R (5′-AGGTTCCTCAGTAGACGTTTCA-3′) and applied to DNA dot blots. Identities of clones were established by sequencing.

Yeast mating assays were performed using constructs and plasmids supplied with the Matchmaker 2 kit (Clontech, methods accord- ing to the manufacturer’s instructions) to eliminate false positive interacting clones.

Antibodies—Mouse monoclonal anti-c-Myc antibody (Clontech) was used at 2 μg/ml. Goat anti-LC2 and anti-GluR2 (Santa Cruz Biotechnol- ogy) antibodies were employed at 0.2 μg/ml. Anti-stargazin extreme C terminus-targeted polyclonal antibodies were raised against custom-synthesized peptide (Cytopeia, Seattle, WA) was used at 1 μg/ml. The column eluate was collected, and the columns subsequently washed with 10 ml of buffer A as above and then the respective peptide, used as antigen to raise the anti-stargazin antibodies coupled to the immunofluorescence columns, was applied as for the non-specific peptide above. After 12 h of circulation through the column, the column was subjected to acid-elution protocol as above. The column was eluted with 10 ml of Buffer A supplemented with sucrose (10%, w/v) and Triton X-100 (1%, v/v) before being subjected to the acid-elution protocol above to elucidate whether all proteins had been displaced from the column and to displace peptide so the columns could be used again.

Acid Elution—Proteins were eluted with acid elution buffer comprising 50 mM glycine pH 2.3, 500 mM KCl, 10% (w/v) sucrose, 1% (v/v) Triton X-100. 1-ml fractions were collected in Eppendorf tubes and immediately neutralized with 1 x Tris.

Peptide Elution—To test for the specificity of association of proteins with the immunofluorescence columns and specificity of elution, peptide whose sequence matched part of the extracellular domain of stargazin (CTKT5SVESENST) and thus not competitive for the stargazin-anti- stargazin antibody binding sequences on the two different anti-starga- zin antibody immunofluorescence columns was applied (0.5 μg, 4 μl), with circulation, to the immunofluorescence columns in buffer A supplemented with 0.5% (v/v) Triton X-100. Filters were washed four times with 10 ml of Buffer A supplemented with sucrose (10%, w/v) and Triton X-100 (1%, v/v) before being subjected to the acid-elution protocol above to elucidate whether all proteins had been displaced from the column and to displace peptide so the columns could be used again.

Immunoprecipitations—Stargazin and associated proteins purified by immunofluorescence column elution as described above was prepared for immunoprecipitation studies by first incubating with the stargazin C-terminal peptide (0.5 μM) to compete out the interac- tion of stargazin with the anti-stargazin antibody that leached off the immunofluorescence column by the acid elution protocol. This preparation
X-100 solubilized cerebellar membranes were combined with 10–20 μl of anti-LC2, anti-stargazin or anti-GluR2 antibodies, in 1-ml reactions of IP buffer (10 mM HEPES pH 7.5, 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1% (v/v) Triton X-100) plus 10% (w/v) sucrose.

In the immunoprecipitation reaction, purified stargazin or Triton X-100 solubilized cerebellar membranes were combined with 10–20 μl of anti-LC2, anti-stargazin or anti-GluR2 antibodies, in 1-ml reactions in IP buffer with protease inhibitors (aprotinin, 2 μg/ml; pepstatin A, 1 μg/ml; leupeptin 1 μg/ml, Sigma) overnight at 4 °C. Samples were incubated with EZview Red protein G affinity gel, pelleted, washed, and the protein complexes eluted with 50 mM glycine pH 2.5. Neutralized samples were prepared for immunoblotting.

Protein Determination—Protein concentrations were determined according to the method of Lowry et al. (26) employing bovine serum albumin as standard for calibration.

RESULTS

Yeast Two-hybrid Screen: the C Terminus Stargazin Bait Interacts with Both PDZ and Non-PDZ Domain-containing Proteins—The fidelity of PCR amplification of our stargazin bait was confirmed by DNA sequencing. We confirmed that we had subcloned the bait sequence in-frame by in vitro translat-ting the construct and analyzing the product by immunoblotting using antibodies recognizing the N terminus e-Myc sequence and the extreme C terminus of stargazin. A unique immunopositive band was identified with both antibodies that had the identical, predicted molecular size (21 kDa). These bands were not detected when empty vector was analyzed in the same way (Fig. 1). The bait sequence is depicted in Fig. 2A.

To identify stargazin C terminus interacting proteins we screened a mouse brain cDNA library with the C-terminal bait. A total of 5.5 × 10⁶ clones were screened. By low stringency auxotrophic challenge, 600 interacting clones were identified. Following re-screening on high stringency media, the number of positive interacting clones was reduced to 129. Identical clones were identified by restriction enzyme digest analysis and some overlapping clones were identified by dot blot hybridizations (data not shown). Since the extreme C-terminal amino acid sequence of stargazin confers a consensus high affinity binding site for PDZ domain-containing proteins we used a PSD-95 hybridization probe in dot blot screens to identify the presence of PDZ domain containing clones. PDZ domain-containing proteins were indeed identified among the group of strong interactors. Sequence analysis revealed the identity of the clones, and a list of proteins represented by 34 independent clones is shown in Table I. Following yeast mating analyses (see “Experimental Procedures” Clone Analysis), three of these strong positive interacting clones, representing Na⁺/K⁺ ATPase β-polypeptide, axonal-associated cell adhesion molecule (BIG-2) and protein phosphatase-3, were eliminated from further studies as these failed this secondary round of screening. Those strong interactors that passed the yeast mating screens comprised the PDZ domain-containing proteins SAP97, SAP102, and PIST. Two proteins that do not contain PDZ domains were also recognized as stargazin interactors. Three clones of microtubule-associated protein 1A light chain 2 (LC2) were identified as strong interactors. A single clone of microtubule-associated protein 1B light chain 1 (LC1) was also identified and also survived the yeast mating secondary screening analyses, however, based on the time this clone took to grow and express α-galactosidase activity we considered this to be a weaker interaction.

Stargazin Interacts with the Light Chains of Microtubule-associated Proteins—Sequence analysis of the three stargazin-interacting LC2 clones and the single LC1 clone revealed that association was through a common conserved sequence of their light chain (LC) regions. The shortest LC2 clone identified from the screen extended from amino acid residue 2550 to 2774 (Fig. 2B), and the only LC1 clone to be pulled out of the library screen extended from residue 2233, equivalent to residue 2570 of LC2 when their sequences are aligned (16), to residue 2774. As the LC1 clone was twenty residues shorter than the shortest LC2 domain, we assumed these residues were unlikely to contribute to the interacting domain and therefore felt justified in omitting the 12 base pairs coding for the first four amino acids of LC2, thus making it easier to create the LC construct for in

![Image](http://www.jbc.org/)
corresponding to that of the LC2 construct, i.e. the stargazin C terminus construct interacted with the LC2 domain (Fig. 3B, lane 1). Likewise, when this was performed in reverse, i.e. the LC2 protein was used to overlay both the full-length and truncated stargazin bait proteins, anti-LC2 antibody immunoreactivity was identified at the mass of the stargazin constructs (Fig 3B, lanes 2 and 3), confirming that the stargazin C terminus protein and the LC2 domain specifically and directly interact with each other outside of the yeast system and do so independently of the PDZ domain-interacting sequence of stargazin. Lanes 4 and 5 of Fig. 3B are the silver-stained counterparts of lanes 2 and 3 in Fig. 3B, thus identifying the total protein composition of these samples. Proteins other than the stargazin constructs (components of the E. coli expression system) are present in amounts comparable to the stargazin structures, however, these are not highlighted by the anti-LC2 antibody on the gel overlay (lanes 2 and 3 of Fig. 3B) providing further evidence that the stargazin-LC2 interaction is specific.

Light Chain 2 and GluR2 Co-purify with Stargazin ex Vivo—Cerebellar membranes from control mice (+/+) and stargazer mice (stg) were probed by immunoblotting with our extreme C terminus-directed anti-stargazin antibody. A broad, differentially glycosylated protein band of expected size range (37–41 kDa) was identified in +/+ but was totally absent in stg mouse cerebellar membranes (Fig. 4A, Input). We confirm that stargazer mice are null for stargazin. Two other prominent anti-stargazin antibody immunopositive species with M, values of ~50 and ~55 kDa were also detected. These were present in both +/+ and stg cerebellar membranes. The comparable intensities of these bands on immunoblots implied that their expression was unaffected by the mutation. When we investigated the levels of AMPA receptor subunits expressed in the cerebellum (+/+ versus stg mice by quantitative immunoblotting; however, we found that GluR2 immunoreactivity was dramatically decreased in stg, being only 27 ± 5% (p < 0.001) of +/+ when normalized against β-actin levels (see Fig. 4B, Input). GluR1 (74 ± 7%; p < 0.05) and GluR4 (69 ± 7%; p < 0.05) were also down-regulated (data not shown).

Immunopurification of Stargazin: Co-association of GluR2—Cerebellar membranes from +/+ and stg mice were solubilized in Triton X-100. Fig. 4A shows that stargazin (indicated by the arrow) was present in both the Triton X-100 soluble and insoluble fractions from +/+, the larger fraction being insoluble, none was found in the column flow through fraction showing that all the soluble stargazin was extracted by the extreme C terminus directed anti-stargazin antibody immunoaffinity column. Acid-eluted fractions 2, 3, and 4 were enriched for stargazin (Fig. 4A, lanes 2–4). The ~55-kDa band (but not the 37–41 kDa stargazin species) was also purified from Triton X-100 soluble material derived from both +/+ and from stg mice (Fig. 4A). When this material was probed for GluR2, GluR2 was found to reside in the Triton X-100 insoluble and soluble fractions from both +/+ and stg cerebellar, though it was clear that a greater proportion was insoluble in the +/+ versus stg cerebellum. Furthermore, a large proportion of Triton X-100 soluble GluR2 applied to the anti-stargazin immunoaffinity columns was recovered in the column flow through (Fig. 4B, FT). However, Triton X-100 soluble GluR2 from +/+ but not stg cerebella was retained on the immunoaffinity column (Fig. 4B) confirming that stargazin interacts with AMPA receptor subunits. Furthermore, this shows that the purified ~55-kDa band was not an AMPA receptor subunit-interacting protein. Stargazin (37–41 kDa) was also purified using our TM4 proximal C-terminal-directed anti-stargazin antibody immunoaffinity column, in this case the ~55-kDa band was not detected but GluR2 was present in this preparation further endorsing that.
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FIG. 4. Immunoaffinity purification of stargazin and its associating proteins. A, immunoblot analysis of the anti-stargazin antibody immunoreactive species throughout the purification assay. Left and right panels are immunoblots probed with the extreme C terminus directed anti-stargazin antibody. Input is cerebellar membranes from control (+/+) and stargazer (stg) mice. Insol refers to that proportion of +/- (left panel) and stg (right panel) immunoreactive material that remained in the insoluble fraction. Sol refers to the Triton X-100 soluble fraction that was applied to the immunoaffinity columns. FT refers to column flow-through, equivalent to the soluble material that did not bind to the column. The purified material that was acid-eluted from the column is referred to as Output, pH-eluted fractions 1–4. The bold arrow indicates the stargazin band that is absent from material obtained from stargazer mice. B, GluR2 solubilization and retention on the anti-stargazin immunoaffinity column. The immunoblot was probed with anti-GluR2 antibody. Input is 10 μg of cerebellar membranes from control (+/+) and stargazer (stg) mice. Note reduced expression level of GluR2 in stg (27 ± 5% of +/- level; p < 0.001). Output is material from fraction 2 that had been eluted from the immunoaffinity columns probed for GluR2. Triton X-100 soluble and insoluble highlights the differential solubility of GluR2 from +/- and stg cerebellar. Column FT is GluR2 that failed to bind to the immunoaffinity columns. C, LC2 is co-eluted with stargazin. Cerebellar membranes (memb, 10 μg) and purified stargazin fraction 2 (frac 2) were probed for GluR2 (left) and LC2 (right). D, PSD-95, SAP97, and β-actin were not present in the stargazin-purified material. Cerebellar membranes from +/- and stg (memb, 10 μg) or just from +/- mice (M, 10 μg) and Triton X-100 soluble extract (soluble) and fraction 2 from the respective immunoaffinity columns were screened for the presence of PSD-95, SAP97, and β-actin. Note bands at ~53 kDa in the fraction 2 samples when probed for β-actin. These are the heavy chains of antibody that routinely leach from the immunoaffinity columns during their first few uses. These bands appeared when primary antibody was omitted from the assay as a result of detection by the secondary anti-rabbit IgG-HRP.

GluR2 interacted with stargazin (data not shown).

Immunopurified Stargazin Co-associates with LC2—Purified stargazin from both the extreme C-terminal anti-stargazin antibody immunoaffinity column (Fig. 4C) and the TM4 proximal anti-stargazin immunoaffinity column (data not shown) was also found to contain LC2, demonstrating that stargazin also co-associates with LC2 in vivo. Triton X-100 soluble material from +/- cerebella was also applied to a mock immunoaffinity column devoid of coupled antibodies. The column was subjected to the same process used for immunoaffinity column purification as above. LC2, GluR2, and stargazin were not detected in the acid-eluted fractions confirming the specificity of the interaction (data not shown).

PSD-95 and SAP97 were retained entirely in the Triton X-100 insoluble cerebellar membrane fraction and not associated with purified stargazin (Fig. 4D). Although β-actin was partially solubilized in Triton X-100 it was not retained on the anti-stargazin affinity column (Fig. 4D).

Does LC2 Form a Tripartite Complex with Stargazin and GluR2 in Vivo?—Using our purified stargazin preparation we then investigated whether stargazin-LC2 and stargazin-GluR2 associated independently of each other or whether there was a fraction that was co-assembled into a tripartite complex, in vivo. Using Triton X-100 soluble material from +/- cerebellar we performed immunoprecipitation (IP) assays using anti-GluR2, anti-stargazin and anti-LC2 antibodies to pull-down interacting proteins (see Fig. 5). LC2 was detected in the material immunoprecipitated by both the anti-GluR2 and anti-stargazin antibodies. This was further confirmation of the LC2-stargazin association ex vivo but also evidence that LC2 interacted with GluR2.

To investigate whether GluR2 associated with LC2 through stargazin thus forming a tripartite complex we decided to perform IP studies on our extreme C terminus anti-stargazin antibody immunopurified material. The rationale being that if a tripartite complex existed then performing IPs on the purified stargazin preparation using anti-GluR2 antibody to pull down GluR2 would co-precipitate LC2 and vice versa. However, this approach was complicated by the fact that we had observed that a minor fraction of anti-stargazin antibody used to generate the immunoaffinity column was displaced by the acid-elution step. Fig. 4D, frac 2, a 53-kDa band that is IgG heavy chains was detected when immunoblots were probed with anti-rabbit IgG-HRP alone without previous exposure to primary antibodies. This potentially could have compromised our interpretation of the immunoprecipitation studies that were designed to test whether LC2-stargazin-GluR2 complexes existed. To circumvent this problem we competitively antago-
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Fig. 5. Immunoblot analysis of immunoprecipitated proteins. The antibodies used in the immunoprecipitations are shown above the panel. C is a no-antibody control. The products of each IP were probed with anti-LC2. The LC2 protein was precipitated by all three antibodies (indicated by the arrows) from both Triton X-100-solubilized cerebellar membranes and from the purified stargazin complex.

...recognized the stargazin-anti-stargazin antibody interaction that may exist in the purified sample by applying the immunogenic peptide used to generate the antibody (see “Experimental Procedures”). The precleared, dialyzed fraction 2 was subjected to immunoprecipitations using anti-GluR2 and anti-LC2 antibodies as immunoprecipitants. The immunoprecipitation material was then analyzed by immunoblotting. The LC2 antibody immunoprecipitated LC2 as well as stargazin and GluR2 (Fig. 5). Likewise, anti-GluR2 antibody immunoprecipitated GluR2, stargazin, and LC2 (data not shown) confirming that at least a fraction of stargazin in the immunopurified extract existed in a tripartite complex comprising LC2, stargazin, and GluR2, the first observation that these three proteins co-associate in cerebellum. LC2, stargazin, and GluR2 were not pulled down when the immunoprecipitation antibody was omitted from the assay (Fig. 5, C).

Immunopurification of Stargazin: Peptide Elution—In order to substantiate our immunoprecipitation observations we immunopurified stargazin as before except displaced bound stargazin and its associating proteins from the immunoaffinity column by competitive displacement with immunogenic peptide (see “Experimental Procedures”). Under these conditions antibody was not displaced from the column (Fig. 6).

Using this method only a fraction (~60%) of stargazin but all the GluR2 bound to stargazin was eluted. In contrast only a minor fraction of LC2 bound to stargazin was eluted. The residual column bound stargazin and LC2 was subsequently acid-eluted (Fig. 6). This implies that stargazin can engage in a complex with LC2 as a bipartite complex and does not require association with GluR2. This may have implications for the temporal order in which these proteins interact and/or dissociate in vivo.

Discussion

We have used the intracellular C-terminal tail of mouse stargazin as bait in a yeast two-hybrid (Y2H) assay to screen a mouse brain cDNA library for stargazin-interacting proteins. Strong interactors included the PDZ domain-containing proteins SAP97, SAP102, and PIST in accordance with the fact that the extreme C terminus of stargazin bears a classic consensus sequence (~RRTTPV) for interaction with the PDZ domain-containing proteins. Chen et al. (13) and Dakoji et al. (27) have also reported that SAP97 and SAP102 interact in Y2H with the cytosolic tails of stargazin and the γ4 isoform of stargazin, respectively. However, these appear not to interact in vivo (27). PSD-95 and PSD-93 have also been shown to be stargazin-interacting proteins by Y2H though in this case they do interact in vivo (13, 27). We did not pull out PSD-95 or PSD-93 in our Y2H screen though it is quite possible that our selection strategy filtered out these interactors. Our stargazin-purified cerebellar extract was also found to be devoid of SAP97 and PSD-95, though this might have been expected as these proteins are largely found in the Triton X-100 insoluble postsynaptic density (PSD) fraction (28, 29). Alternatively, it might be argued that a complex of interacting proteins built around the PDZ domain interacting sequence would hinder access of the C terminus-targeted anti-stargazin antibody that we used to generate the immunoaffinity column. Thus, theoretically we might have selectively purified a subpopulation of stargazin whose PDZ-protein-interacting domain was unoccupied. However, we believe this not to be the case as our immunoaffinity purification columns were found to be 100% efficient in their capacity to extract Triton-soluble stargazin. None was detected in the column flow through, thus we believe if PDZ proteins such as PSD-95 and SAP97 are associated with stargazin in this fraction we would have been able to detect them in immunoblots. We are confident therefore that our purified material represents stargazin and associated proteins that reside outside of the PSD. We have subsequently used sodium deoxycholate and SDS to sequentially solubilize and purify residual Triton X-100-insoluble stargazin to investigate synaptic stargazin-associated proteins.

Two proteins that do not contain PDZ domains, microtubule-associated protein light chain 2 (LC2) and microtubule-associated protein light chain 1 (LC1), were also recognized as bait interactors in our Y2H, the former having higher affinity based on its Y2H performance than the latter. Both survived the yeast mating secondary screening analyses. By gel overlay analysis we have proved that this interaction occurs independently of the extreme RRTTPV sequence of stargazin and furthermore, that the two proteins interact directly with each other.

The sequence homology between LC1 and LC2 (16) and their similarity of function (30) implies that they are likely to have a common stargazin interaction domain. As detailed in Table I, the Y2H screen detected only one LC1 clone but three LC2 clones as stargazin interactors. In addition the LC1 clone, when interacting with stargazin in the yeast mating assay, grew more slowly on high stringency media than the LC2 clones (data not shown). These pieces of evidence together with the established developmental expression profile of LC2 as predominant over LC1 in adult tissue (16) leads us to believe that LC2 might be the predominant interactor with stargazin in adult cerebellum.

Our C terminus-directed anti-stargazin antibody was used to generate two identical stargazin immunoaffinity columns. Two extreme C-terminal anti-stargazin antibody-immunoreactive species, stargazin and a ~55 kDa protein that might be a stargazin isofrom (4, 8, 15) were purified from Triton X-100-solubilized control mouse cerebella. Only the ~55 kDa immunoreactive species was purified from stargazer mouse cere-
bellla. A major fraction of stargazin was retained in the Triton X-100 insoluble pellet, this is in accordance with the role of stargazin as an AMPA receptor synaptic targeting molecule and its ability to interact with the cytoskeleton through synaptic anchoring/clustering proteins e.g. PSD-95 (14), an association that might be anticipated to render these proteins Triton X-100 insoluble. GluR2 was only detected in the immunopurified material from control mice. Stargazin purified on our TM4 proximal C-terminal anti-stargazin antibody immunofluorescence column and screened on immunoblots with our extreme C terminus-directed anti-stargazin antibody was devoid of the ~55-kDa species but did contain GluR2 and LC2. Therefore if the ~55-kDa species from control and stargazer cerebellum is a stargazin isoform we suggest it is not operating as a transmembrane AMPA receptor regulatory protein (TARP, Refs. 15 and 27). Our observation confirms that stargazin is a TARP in brain (15). We have therefore shown that GluR2 interacts with LC2 and thus through it probably microtubules. Secondly, we provide evidence that non-synaptic GluR2 interacts with LC2 through stargazin.

Little is also known about how PSD-95- and/or PSD 93-stargazin-AMPA complexes are tethered to the synaptic cytoskeleton (13, 14, 27), though microtubules may play a role (31). It is intriguing to note that MAP1A, the regulatory subunit of LC2, has been reported to interact with PSD-93 through its GK domain to localize it to dendritic microtubules (31). Concomitant binding of PSD-93 to a substrate through its PDZ domain appears to stabilize this interaction with MAP1A. One such PSD-93 PDZ domain binding substrate could be stargazin (13, 15, 27). Thus, MAP1A may potentially link PSD proteins to the cytoskeleton. CRIP1, a PSD-95 interacting protein induces reorganization of microtubules and acts as bridge for PSD-95 and the tubulin-based cytoskeleton at asymmetric postsynaptic loci (32) so clearly there is previous evidence of microtubule-AMPa receptor interactions.

However, our data imply that AMPA receptors coupled to the stargazin-LC2 complex described herein may be at a premature stage of trafficking/targeting to dendritic microtubules where subsequent interaction of both MAP1A and stargazin with PSD-93 for example may induce a conformational change that may stabilize the complex, tether it to the cytoskeleton and influence AMPA receptor signaling and potentially synaptic plasticity.

A direct interaction of stargazin with the PSD-95 (14) appears to be pivotal to the ability of stargazin to target AMPARs to the synapse. Disruption of this interaction by PKA-phosphorylation (33, 34) or deletion of the YTPV sequence of stargazin would eliminate its ability to direct synaptic targeting of AMPARs (13). The findings in this study identifies microtubule-associate protein 1 light chain 2 as a novel stargazin-associating protein, which interacts with stargazin in brain and does so, at least in part, when stargazin is associated with AMPA receptor subunits prior to synaptic targeting. GluR2 co-associates with the stargazin-LC2 complex when it is in a fully glycosylated state (insensitive to Endo-H but sensitive to N-glycosidase deglycosylation) implying that GluR2 couples to stargazin-LC2 either as it is trafficked from the Golgi to the neuronal surface and/or during migration in the plasma membrane toward the synapse (39, 40). Having identified the interacting domains of stargazin and LC2 we are now endeavoring to dissect the role that this interaction plays in stargazin-mediated events in neurons and possibly glia.

REFERENCES

1. Noebels, J. L., Qiao, X., Bronson, R. T., Spencer, C., and Davison, M. T. (1990) Epilepsy Res. 7, 129–135
2. Letts, V. A., Felix, R., Biddlecome, G. H., Arikath, J., Mahaffey, C. L., Valenzuela, A., Bartlett, P. F., Mori, Y., Campbell, K. P., and Frankel, W. N. (1998) Nat. Genet. 19, 340–347
3. Ives, J. H., Drewery, D. L., Twiari, P., and Thompson, C. L. (2000) Eur. J. Neurosci. 12, 1635–1645
4. Sharp, A. H., Black, I. I. L., Dubel, S. J., Sundarrajan, S., Shen, J.-P., Yunker, A. M. R., Copeland, T. D., and McEnery, M. W. (2001) Neuroscience 105, 589–617
5. Qiao, X., Chen, L., Gao, H., Bao, S., Hefti, F., Thompson, R. F., and Knussel, B. (1998) J. Neurosci. 18, 6990–6999
6. Di Pasquale, E., Keegan, K. D., and Noebels, J. L. (1997) J. Neurophysiol. 77, 621–631
7. Kang, M.-G., Chen, C.-C., Felix, R., Letts, V. A., Frankel, W. N., Mori, Y., and Campbell, K. P. (2001) J. Biol. Chem. 276, 32917–32924
8. Krapivker, N., Dai, S., Spach, V., Lazicova, L., Marais, E., Bohn, G., and Hofmann, F. (2000) FEBS Lett. 470, 189–197
9. Rossset, M., Cens, T., Restituito, S., Barrere, C., Black III, J. L., McEnery, M. W., and Charnet, P. (2001) J. Physiol. 532.3, 583–596
10. Green, P. J., Warre, R., Hayes, P. D., McNaughton, N. C., Medhurst, A. D., Pangalos, M., Duckworth, D. M., and Randall, A. D. (2001) J. Physiol. 533, 467–478
11. Thompson, C. L., Jalilian Tehrani, M. H., Barnes, E. M., and Stephenson, F. A. (1998) Mol. Brain Res. 60, 282–290
12. Chen, L., Bao, S., Qiao, X., and Thompson, R. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12132–12137
13. Chen, L., Chetkovich, D. M., Petralia, R. S., Sweeney, N. T., Kawasaki, Y., Wenthold, R. J., Bredt, D. S., and Nicoll, R. A. (2000) Nature 408, 938–943
14. Schnell, E., Szemere, M., Kitzmizadegan, S., Chen, L., Bredt, D. S., and Nicoll, R. A. (2002) Proc. Natl. Acad. Sci. 99, 13902–13907
15. Tomita, S., Chen, L., Kawasaki, Y., Petralia, R. S., Wenthur, R. J., Nicoll, R. A., and Bredt, D. S. (2000) J. Cell Biol. 141, 805–816
16. Langkopf, A., Hammarback, J. A., Muller, R., Valle, R. B., and Garner, C. C. (1992) J. Biol. Chem. 267, 16561–16566
17. Tegel, M., Wiche, G., and Propst, F. (1999) J. Cell Biol. 143, 695–707
18. Mei, X., Sweett, A. J., and Hammarback, J. A. (2000) Brain Res. Bulletin 53, 801–806
19. Qiao, X., Hefti, F., Knussel, B., and Noebels, J. L. (1999) J. Neurosci. 19, 640–648
20. Hashimoto, K., Fukaya, M., Qiao, X., Sakimura, K., Watanabe, M., and Kano, M. (1999) J. Neurosci. 19, 6027–6036
21. Thompson, C. L., and Stephenson, F. A. (1994) J. Neurochem. 62, 2037–2044
22. Gietz, D., StJean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
23. Qiao, X., Drewery, D. L., and Thompson, C. L. (2002) J. Neurochem. 80, 317–327
24. Wu, L., Davies, S. L., North, P. S., and Goulaouic, H. (2000) J. Biol. Chem. 275, 8365–8368
25. Kennenberg, K., Baur, R., and Sigel, E. (1997) J. Neurochem. 68, 1352–1360
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
27. Dakoji, S., Tomita, S., Karimzadegan, S., Nicoll, R. A., and Bredt, D. S. (2003) Neuropharmacology 45, 849–856
28. Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1992) Neuron 9, 929–942
29. Valtschanoff, J. G., Burette, A., Davare, M. A., Leonard, A. S., Heil, J. W., and Weinberg, R. J. (2000) Eur. J. Neurosci. 12, 3605–3614
30. Noiges, R., Eichinger, R., Kutschera, W., Fischer, I., Nemeth, Z., Wiche, G., and Propst, F. (2002) J. Neurosci. 22, 2106–2114
31. Brenman, J. E., Topinka, J. R., Cooper, E. C., McGee, A. W., Rosen, J., Milroy, T., Ralston, H. J., and Bredt, D. S. (1998) J. Neurosci., 18, 8805–8813
32. Niethammer, M., Valtschanoff, J. G., Kapur, T. M., Allison, D. W., Weinberg, T. M., Craig, A. M., and Sheng, M. (1998) Neuron 20, 693–707
33. Chetkovich, D. M., Chen, L., Stocker, T. J., Nicoll, R. A., and Bredt, D. S. (2002) J. Neurosci. 22, 5791–5796
34. Choi, J., Ko, J., Park, E., Lee, J. R., Yoon, J., Lim, S., and Kim, E. (2002) J. Biol. Chem. 277, 12359–12363
35. Ikeda, A., Zheng, Q. Y., Zuberi, A. R., Johnson, K. R., Naggert, J. K., and Nishina, P. M. (2002) Nat. Genet. 30, 401–405
36. Morris, J. A., Kandpal, G., Ma, L., and Austin, C. P. (2003) Hum. Mol. Genet. 12, 1591–1608
37. Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J., and Olsen, R. W. (1999) Nature 397, 69–72
38. Wang, H., and Olsen, R. W. (2000) J. Neurochem. 75, 644–655
39. Malinow, R., and Malenka, R. C. (2002) Annu. Rev. Neurosci. 25, 103–126
40. Bredt, D. S., and Nicoll, R. A. (2003) Neuron 40, 361–379
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