The α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate Receptor Trafficking Regulator “Stargazin” Is Related to the Claudin Family of Proteins by Its Ability to Mediate Cell-Cell Adhesion*

Received for publication, January 18, 2005, and in revised form, March 9, 2005
Published, JBC Papers in Press, March 10, 2005, DOI 10.1074/jbc.M500623200

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Mutations in the Caeng2 gene encoding the neuronal transmembrane protein stargazin result in recessively inherited epilepsy and ataxia in “stargazer” mice. Functional studies suggest a dual role for stargazin, both as a modulatory γ subunit for voltage-dependent calcium channels and as a regulator of post-synaptic membrane targeting for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptors. Co-immunoprecipitation experiments demonstrate that stargazin can bind proteins of either complex in vivo, but it remains unclear whether it can associate with both complexes simultaneously. Caeng2 is one of eight closely related genes (Caeng1-8) encoding proteins with four transmembrane segments, cytoplasmic termini, and molecular masses between 25 and 44 kDa. This group of Caeng genes constitutes only one branch of a larger monophyletic assembly dominated by over 20 genes encoding proteins known as claudins. Claudins regulate cell adhesion and paracellular permeability as fundamental components of non-neuronal tight junctions. Because stargazin is structurally similar to claudins, we hypothesized that it might also have retained claudin-like functions inherited from a common ancestor. Here, we report that expression of stargazin in mouse L-fibroblasts results in cell aggregation comparable with that produced by claudins, and present evidence that the interaction is heterotypic and calcium dependent. The data suggest that the cell adhesion function of stargazin preceded its current role in neurons as a regulator of either voltage-dependent calcium channels or AMPA receptors. We speculate these complexes may have co-opted the established presence of stargazin at sites of close cell-cell contact to facilitate their own evolving intercellular signaling functions.

Among the four subunits comprising the mammalian voltage-gated Ca\(^{2+}\) channel (α1, α2δ, β, and γ), the γ subunit is the least well understood. Originally copurified with the 1,4-dihydropyridine receptor (L-type Ca\(^{2+}\) channel) from skeletal muscle (1–3), early studies focused exclusively on the ability of the glycosylated 32-kDa transmembrane γ subunit to modulate the electrophysiological properties of the channel. Functional expression of the γ subunit with other channel subunits in Xenopus oocytes produced minor and variable effects on the amplitude of the resulting Ca\(^{2+}\) currents (4, 5). In 1998, Letts and colleagues (6) used positional cloning to identify the defect responsible for epilepsy and ataxia in the stargazer (stg) mutant mouse. The novel gene encoded a glycosylated 36-kDa neuronal protein (stargazin) with 25% amino acid identity to the skeletal muscle Ca\(^{2+}\) channel γ subunit. Expression of stargazin protein with neuronal Ca\(^{2+}\) channel α1A, β1, and α2δ1 subunits in cultured baby hamster kidney cells inhibited Ca\(^{2+}\) currents to a small, but significant, degree by altering the balance of channel availability and inactivation. These subtle modulatory effects were reminiscent of the behavior of the skeletal muscle γ subunit (4, 5). It was concluded that stargazin was a neuronal Ca\(^{2+}\) channel γ subunit (γ\(_{2}\), gene symbol Caeng2), complementing the skeletal muscle-specific γ1 subunit (Caeng1) (6), and other possible functions went largely unexplored. The role of stargazin as a Ca\(^{2+}\) channel subunit was supported by studies that demonstrated that γ\(_{2}\) protein could be copurified and communoprecipitated with other channel subunits from rodent brain and could modulate the biophysical properties of Ca\(^{2+}\) channels composed of different subunit isoform combinations (7–9).

In 2000, the primary identity of γ\(_{2}\) as a voltage-gated Ca\(^{2+}\) channel subunit was challenged by discovery of a novel function, when it was found to be essential for the membrane localization and synaptic targeting of AMPA-type glutamate receptors (AMPArs) in mouse cerebellar neurons (10). Stargazer mutant mice, which exhibit gross defects in cerebellar function, lack detectable AMPAR currents in granule neurons. Transfection of Caeng2 into cultured stg/stg granule cells completely restored synaptic AMPAR function. Other experiments demonstrated that γ\(_{2}\) coimmunoprecipitates with GluR1, -2, and -4 AMPAR subunits expressed in heterologous COS cells, and that the postsynaptic density protein, PSD-95, is required to translocate the γ\(_{2}\)-AMPA receptor complex to neuronal postsynaptic membranes (10). Interestingly, this investigation failed to identify the Ca\(^{2+}\) current abnormalities in stg/stg neurons that might have been predicted from in vitro studies of γ\(_{2}\) function as a Ca\(^{2+}\) channel subunit. This could be explained if loss of γ\(_{2}\) is functionally rescued by compensating proteins or, alternatively, if γ\(_{2}\) is not required as a Ca\(^{2+}\) channel subunit in vivo. Subsequent analyses extended these results to support a central role for γ\(_{2}\) in the AMPA receptor pathway (11–17).

* This work was supported by National Institutes of Health Grant NS042632 (to D. L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Caeng or CACNG, Ca\(^{2+}\) channel γ; LIM, lens intrinsic membrane; EMP, epithelial membrane protein; PMP, peripheral myelin protein; CLDN or Cldn, claudin; GFP, green fluorescent protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; MDCK, Madin-Darby canine kidney; TJ, tight junction; CAM, cell adhesion molecule; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor.
Beginning in 1999, our laboratory and others identified six novel genes with homology to Caenog1 and -2 (18–20). Caenog3–8 are all expressed in fetal and adult brain and some members of this group, Caenog4–7, are transcribed in a wide variety of additional tissues (7, 20–24). Recent functional analyses demonstrated that the novel γ subunit-like proteins can, like γ2, interact with and regulate voltage-gated Ca2+ channels, AMPARs, or both, however, the results were quite variable. Klugbauer et al. (7) reported that γ2 altered the steady state inactivation of the P/Q-type Ca2+ channel although γ2 accelerated the kinetics of current activation and inactivation of the T-type calcium channel in HEK-293 cells (7). Rousset et al. (9) showed that γ2 hyperpolarized the activation and increased the inactivation of P/Q-type Ca2+ channels expressed in Xenopus oocytes, with some dependence on other auxiliary channel subunits. Kang et al. (8) demonstrated that γ2 copurified with other neuronal Ca2+ channel subunits in vivo. Coexpression of γ2 protein with the pore forming Ca2,2.2 (α3b) protein nearly eliminated N-type Ca2+ currents in Xenopus oocytes or COS cells, and reduced conductance through L-type and P/Q-type Ca2+ channels. Unusually, the mechanism of inhibition in this case appeared to be a reduction of α3 subunit protein expression by γ2 rather than modulation of existing channels (25). Hansen et al. (26) showed that coexpression of γ2 significantly decreased currents through recombinant T-type Ca2+ channels (Ca3.1) in HEK-293 cells and native T-type Ca2+ channels in atrial HL-1 cells, and that this effect did not accompany any decrease in the level of the Ca3.1 mRNA or protein. The abilities of γ1–γ6 to regulate AMPA receptors appear to be more straightforward than their regulation of voltage-gated Ca2+ channels: γ3, γ4, and γ6 each bind to PSD-95 and appear to function much like γ2 in regulating AMPARs, whereas there is no evidence yet of such a role for γ1, γ3, or γ5 (17, 27). As a result, some have relabeled γ2, γ3, γ4, and γ6 as “transmembrane AMPA-receptor regulatory proteins,” or TARPs (27).

The expansion of protein families through gene and whole genome duplication provides raw material for adaptive evolution via mutation and selection, and is considered a driving force in the development of biological novelty and complexity (28–30). Whereas the selective pressures that determine the fate of recently copied genes are poorly understood, theoretical models propose alternative outcomes. In some models, duplicated genes evolve novel adaptive functions while losing ancestral functions; in other models, the ancestral functions are retained as new ones emerge (29–31). This raises the intriguing possibility that the evolution of voltage-gated Ca2+ channel modulation and AMPAR trafficking functions for the γ subunit proteins did not occur at the expense of some fundamental ancestral capability. The expression of γ subunit genes in several tissue types that do not express either AMPARs or voltage-gated Ca2+ currents is consistent with such a third function.

We previously reported a phylogenetic relationship between Ca2+ channel γ subunits and a larger family of proteins called claudins (18). The claudin superfamily also contains peripheral myelin protein-22 (PMP-22), lens intrinsic membrane protein-2 (LIM-2), and epithelial membrane proteins 1 through 3 (EMP1–3). Claudins are cell adhesion molecules that selectively regulate paracellular permeability at epithelial tight junctions (TJs) and facilitate other types of inter- and intramembranous interactions in nonepithelial cells (32, 33). There is evidence that PMP-22 and LIM-2 may have similar roles in these or other cell types (34, 35). Based on the phylogenetic relationship and structural similarity of γ subunits and claudins, and applying current models of adaptive evolution among duplicated genes, we speculated that these proteins might yet share a basic ancestral function. Here, we test the hypothesis that the neuronal protein, stargazin, can mediate cell adhesion like the tight junction protein, Claudin-1. We present evidence in support of this hypothesis and discuss the potential implications in terms of Ca2+ channel and AMPA receptor behavior in neurons.

**Experimental Procedures**

**Phylogenetic Analysis of Claudin Family Proteins**—Amino acid sequences of the human proteins EMP1 (gi:3044368), EMP2 (gi:17068643), EMP3 (gi:17066644), LIM2 (gi:13445660), PMP-22 (gi:268803), CACNG1 (gi:1168946), CACNG2 (gi:5174405), CACNG3 (gi:5729756), CACNG4 (gi:10719940), CACNG5 (gi:22027551), CACNG6 (gi:22027554), CACNG7 (gi:21361975), CACNG8 (gi:24586866), CLDN1 (gi:6685283), CLDN2 (gi:12229749), CLDN3 (gi:4502875), CLDN4 (gi:4502877), CLDN5 (gi:4502879), CLDN6 (gi:11141665), CLDN7 (gi:10835008), CLDN8 (gi:6685307), CLDN9 (gi:11141861), CLDN10 (gi:5921465), CLDN11 (gi:10938016), CLDN12 (gi:6685308), CLDN14 (gi:6685304), CLDN15 (gi:6685306), CLDN16 (gi:5729970), CLDN17 (gi:6685309), CLDN18 (gi:7387578), CLDN19 (gi:22507372), CLDN20 (gi:18088580), CLDN23 (gi:47605352), and CLARIN1 (gi:28144910) were retrieved from GenBankTM. To facilitate alignment, sequences were trimmed to include only the region spanning the first through the fourth transmembrane domains, as predicted by the TMPred program (36). The sequences were aligned using the ClustalW algorithm set to the default parameters within the MegAlign module of the Lasergene (DNASTAR Inc., Madison, WI) software package, viz. gap penalty, 10.00; gap length penalty, 0.20; delay divergent sequences, 30; DNA transition weight, 0.50; and protein weight matrix, Gonnet series. A phylogenetic tree based on this alignment was constructed with the MegAlign program using the same parameters. The linear alignment created by ClustalW was optimized by visual inspection, and shaded so that black represents ≥67% conservation throughout the entire family and gray represents ≥50% conservation within the clade.

Claudin-1, Claudin-2 (Stargazin), and Mutant Claudin-2-N48Q Expression Plasmids—Two types of expression plasmids were constructed: one encoding expression of enhanced green fluorescent protein (EGFP) and Claudin-1, Caecn2, or Caecn2-N48Q protein, and the other for expression of GFP fused to the NH2 terminus of these proteins. cDNAs for mouse Claudin-1 (Cldn1; locus AF072127) (43) and γ2 Caecn2, locus AF077739 (6) were created by reverse transcriptase-PCR of total RNA from adult C57BL/6J mouse brain. Restriction sites and optimized Kozak consensus sequences were appended to PCR amplification products using 5'-GCCATGCGCAA-3' and 5'-TCTTGGCTCACATAC-3' for Cldn1, and 5'-ATGGGGGCTTGGTATGAC3' and 5'-TCTTGCATCGGCAGGTC-3' for Caecn2 cDNA. The cDNAs were cloned into pGEM T-Easy (Promega Corp., Madison, WI) and sequenced to confirm the absence of mutations (Sequwright Laboratories, Houston, TX). The Caecn2-N48Q mutation construct was created using the QuickChange site-directed mutagenesis kit (Stratagene, CA). The cDNAs were then transferred into vectors pRES52-EGFP and pEGFP-C1 (BD Biosciences, Palo Alto, CA), which provided a neomycin resistance selectable marker.

**Cell Lines and Transfections**—L929 mouse fibroblasts (L-cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells were grown at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and lacking antibiotics except during selection of transformed clones. Cells were transfected with plasmids using FuGENE-6 (Roche Diagnostics Corp., VA). All cells were grown at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and lacking antibiotics except during selection of transformed clones. Cells were transfected with plasmids using FuGENE-6 (Roche Diagnostics Corp., Indianapolis, IN) and stable transfecant cells were selected by growth in medium containing 500 μg of G418/ml. Colonies were selected by dilution into 96-well plates. Colonies derived from single cells were expanded and aliquots were frozen in liquid nitrogen. Transient transfections of subconfluent HEK-293 cells in 60-mm culture dishes were done using 2 μg of each DNA construct for a GFP fusion protein and FuGENE-6 reagent, according to the manufacturer’s instructions. Cells were harvested or examined by fluorescence microscopy 48 h later.

**Cell Aggregation Assays**—Subconfluent cells were harvested from culture dishes by treatment with 0.05% trypsin, 0.53 mM EDTA for 10 min, dissociated by gentle pelleting, and washed once with culture medium. 1 × 106 cells in 1 ml of culture medium were placed into duplicate wells of 12-well culture plates, which were precoated with a 1% Noble agar in culture medium gel to prevent cell adherence to the dish. Independent plates were prepared for each time point, equilibrated for 10 min at 37 °C in 5% CO2, sealed, and placed in a gyratory shaker at 80 rpm at 37 °C. Aggregation was arrested and preserved by adding glutaraldehyde to a final concentration of 4%. Cells were diluted
10-fold into balanced electrolyte solution and the particles from each well were counted using a Z1 particle counter (Beckman Coulter Inc., Fullerton, CA) to a particle size threshold of 9 μm. To test the role of Ca\(^{2+}\) in aggregation, EGTA was added to both the agar plate-coating solution and the culture medium. The MaxChelator program (www.stanford.edu/~apatton/maxl.html) was used to calculate the EGTA concentration to achieve the desired free calcium concentrations in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The values used for the calculations were: total Ca\(^{2+}\), 1.9175 mM; Mg\(^{2+}\), 0.513 mM; temperature, 37 °C; pH, 7.6; and ionic strength, 0.338 M. The ratios of EGTA concentration to the expected final free calcium concentrations were: 2.0 mM EGTA (0.82 μM Ca\(^{2+}\)), 1.9 mM EGTA (21 μM Ca\(^{2+}\)), 1.4 mM EGTA (0.570 mM Ca\(^{2+}\)), and 0.0 mM EGTA (1.9 mM Ca\(^{2+}\)). The data are expressed as the mean ± S.D. of two independent measurements. Significance was evaluated using a paired, two-tailed Student’s t test.

### RESULTS

**Sequence Comparison and Phylogenetic Analysis**—We initially suggested the relationship between the Ca\(^{2+}\) channel γ1–γ7 subunits and the Claudin-4 protein in a previous study (18) and extend that observation here to include several additional proteins (Fig. 1A). Three distinct clades were discriminated: claudins, gammas, and a third clade containing PMP-22, LIM-2, and EMP1–3. The distant affiliation of claudins to the protein Clarin-1 (gene symbol USH3A), used as the outgroup in our analysis, was suggested by Adato et al. (38). Additional proteins with homology to the claudins include NKG7 (natural killer cell group 7), TM4SF10 (transmembrane 4 superfamily, member 10), PERP (TP53 apoptosis effector), and C3orf4 (chromosome 3, open reading frame 4) (data not shown). The predicted topology of Caecn2 is shown in Fig. 1B and includes cytoplasmic NH\(_2\) and COOH termini and four membrane spanning segments. This basic organization is conserved among all 33 proteins shown in the phylogeny, with the primary source of variation occurring in the length and sequence of the carboxyl tails. The proteins have a mean amino terminus length of 7 residues prior to the first transmembrane segment, except for CACNG6 and CLDN16, which have 40 and 73 amino-terminal residues, respectively. The mean length of the carboxyl termini, following the final predicted transmembrane segment, is 50 amino acids, with a range of 3 (EMP1) to 201 (CACNG8).

The most highly conserved region among all 33 proteins is the first extracellular loop, which contains the claudin family signature motif (Prosite data base accession PDOC01045Q) (39). A complete amino acid alignment of this region is presented in Fig. 1C. The distantly related Clarin-1 was not included in the alignment. Ten residues with ≥65% identity among all 33 aligned proteins define a consensus sequence motif. Of these, the leucine located eight amino acids from the carboxyl end of the loop is least conserved, but the similarity rises to 91% when conservative substitutions are allowed (D/E, K/R, I/L/M/V, S/T, F/W/Y) (40). It is interesting to note that glutamine is the second most common residue (18%) at the position four amino acids from the carboxyl end of the loop, which is occupied by arginine in all other instances (76%) except for the closely related CACNG1 (serine) and CACNG6 (alanine) proteins. This glutamine is the predominant residue at this position in the EMP/PMP/LIM clade. Several other residues are conserved within only one or two of the three clades. At least one consensus site for N-linked glycosylation is present in all eight Caecn proteins and all five EMP/PMP/LIM proteins, although the precise position varies, but this motif is seen in only 2 of 20 claudin proteins (CLDN1, CLDN12). Because the first extracellular loop is the most highly conserved region among all 33 proteins, and this region is critical for mediating the cell adhesion properties of claudins, we hypothesized that it could perform the same function in the Ca\(^{2+}\) channel γ subunits. The stargazer mutant mouse harbors a functionally null allele of Caecn2, and is considered an important model of the neurological disorders ataxia and epilepsy, so we selected this gene for further analysis. The claudin family prototype, Claudin-1, was selected as a positive control for the cell adhesion assays (41).

**Expression of Wild Type and Mutant Caecn2 Protein**—Previous studies have demonstrated the cell adhesion properties of claudins and cadherins utilized mouse L-fibroblasts (L-cells), which exhibit little endogenous cell-cell adhesion (41–43). In preparation for these assays, we established stably transfected L-cell lines expressing GFP and wild type Claudin-1, wild type Caecn2, or mutant Caecn2-N48Q, from a bicistronic vector. Caecn2-N48Q substitutes glutamine for asparagine to disrupt the sole consensus N-glycosylation site of Caecn2 at amino acid position 48, and was created to test a potential role for glyco-
Stargazin is related to the claudin family of tight junction proteins. A, the relationship of stargazin (Cacng2) and other Cacng proteins to the claudins is shown in a phylogenetic tree inferred from the amino acid alignment of the 33 indicated human proteins: Clarin-1, Cacng1–8, EMP1–3, PMP-22, LIM2, and 20 claudins (CLDN). The alignment includes sequences from the beginning of the first through the end.
Caenog2 Promotes Cell-Cell Adhesion Similar to Claudin-1—Although mouse L-fibroblasts do not adhere strongly to one another, and are therefore suitable for assessing whether transfected genes promote cell adhesion, they do adhere well to other substrates. For our aggregation assay, we placed the freshly trypsinized, dissociated cells into culture dishes coated with agar to inhibit cell-substrate association. Cells were incubated in standard culture medium at 37 °C to maximize viability and recovery from trypsinization while they were subjected to rotary shaking for various lengths of time. The results of these assays are presented in Fig. 3. As expected, untransfected L-cells (negative controls) showed very little cell-cell adhesion, whereas Claudin-1 expressing cells (positive controls) exhibited strong aggregation. Cells expressing Caenog2 aggregates in a manner indistinguishable from those expressing Claudin-1. Cells expressing the Caenog2-N48Q mutant did not aggregate and appeared very similar to untransfected cells. Aggregation is represented quantitatively as the decrease in total particle number at a time point (Nf) divided by the particle number at the beginning of the assay (N0), from a starting value of 1.0. The graph of Nf/N0 values for both Claudin-1 and Caenog2 expressing cells began to diverge from untransfected Caenog2-N48Q cells after about 1 h of shaking. By 3.5 h, the Nf/N0 for Claudin-1 (0.42 ± 0.03) and Caenog2 (0.33 ± 0.02) cells dropped significantly (p < 0.002 for each, mean ± S.E., n = 4) relative to the Nf/N0 for untransfected (0.86 ± 0.03) cells. Morphologically, the aggregates initially appeared as small clusters containing 10–30 cells (Fig. 3, B and F), which then combined to make larger clusters (Fig. 3, C, D, and G). The largest aggregates contained hundreds of cells and had a sheet-like appearance, such as the 200 × 750-μm Caenog2-expressing sheet in Fig. 3H. The occasional aggregates formed by untransfected and Caenog2-N48Q cells usually contained two or three, but always less than eight, cells (Fig. 3, I–L). If incubated for >12 h, the Caenog2 or Claudin-1 cells formed a single, stable, disc-shaped aggregate, whereas untransfected or Caenog2-N48Q cells formed only loose clusters that were easily dissociated by gentle shaking (data not shown).

The absence of aggregation by Caenog2-N48Q expressing cells could be explained if this putative glycosylation site mutation prevented the mature protein from localizing to the cell membrane. To test this possibility, we examined the location of the Claudin-1, Caenog2, and Caenog2-N48Q proteins within cells (Fig. 4). As expected, Claudin-1 was correctly targeted to the cell membrane and was concentrated at sites of close cell-cell contact (Fig. 4D). Caenog2 was also targeted to the cell membrane and appeared to concentrate at cell-cell contacts, but not as strongly as Claudin-1 (Fig. 4, A and B). Caenog2-N48Q, although expressed and detected by immunoblotting (Fig. 2), was not located in the cell membrane, but exhibited a punctate distribution within the cytoplasm.

Caenog2-mediated Cell Adhesion Is Ca2+-Dependent—Claudin-1, -2, and -3 were initially reported to possess Ca2+-independent cell adhesion activity in L-cells (41). To determine whether L-cell adhesion mediated by Caenog2 also exhibited this property, we examined the effect of chelating extracellular
Ca\(^{2+}\) in our assay. The addition of EGTA to the assay medium had a strong, concentration-dependent inhibitory effect on the aggregation of Caecn2 expressing cells. Unexpectedly, the aggregation of Claudin-1 expressing cells was also inhibited by EGTA (Fig. 5). Measured as a percent of the maximum aggregation at all EGTA concentrations assayed (2.0, 1.9, 1.4, and 0.0 mM), the addition of EGTA to either 2.0 mM (0.82 \(\mu\)M free Ca\(^{2+}\)) or 1.9 mM (21 \(\mu\)M free Ca\(^{2+}\)) in the medium reduced the aggregation of Caecn2 cells or Claudin-1 cells by \(-80\%\). Specifically, the percent of maximum aggregation in the presence of \(\approx 1.9\) mM EGTA for the Claudin-1 and Caecn2 cell lines (Claudin-1, 23 \(\pm\) 3%; Caecn2, 24 \(\pm\) 8%; mean \(\pm\) S.E., \(n = 4\)) is significantly different (\(p < 0.002\) and \(p < 0.004\), respectively, by paired two-tailed Student’s \(t\) test) than the percent of maximum aggregation in the presence of \(\approx 1.4\) mM EGTA (Claudin-1, 93 \(\pm\) 3%; Caecn2, 93 \(\pm\) 4%). The presence of 1.4 mM EGTA in the medium (0.570 mM free Ca\(^{2+}\)) did not significantly decrease the aggregation of either Caecn2 cells or Claudin-1 cells compared with EGTA-free medium (1.9 mM free Ca\(^{2+}\)).

**Heterotypic Interactions Among Caecn2 Expressing L-cells**—The data presented in Fig. 3 show that Caecn2 expressing L-cells can aggregate with other Caecn2 expressing L-cells, but do not indicate whether these interactions are homotypic (i.e. both cells of an aggregating pair must express Caecn2) or heterotypic (i.e. only one cell of an aggregating pair must express Caecn2). To test whether heterotypic interactions could occur, we performed cell mixing experiments. Untransfected L-cells labeled with PKH26 red fluorescent dye (red L-cells) were mixed with Claudin-1 or Caecn2 expressing cells, distinguished by their bicistronic coexpression of GFP, or with untransfected, unlabeled L-cells, and allowed to aggregate for 4.5 h. Because damaged cells might bind nonspecifically, we first verified that the PKH26 labeling treatment did not affect cell viability (data not shown). The absence of aggregation between red L-cells, or between red L-cells and unlabeled L-cells, is shown in the top panel of Fig. 6. Less than 5% of these L-cells aggregated, and then only in pairs or triplets. This established that the labeling procedure did not induce nonspecific aggregation. However, aggregates that formed from mixtures of red L-cells and either Caecn2 or Claudin-1 cells contained both red and green fluorescent cells, and were similar in size to aggregates formed by Caecn2 or Claudin-1 cells alone. Approximately 80% of the red L-cells aggregated with Claudin-1 (Fig. 6, middle row) or Caecn2 expressing cells (Fig. 6, bottom row). Red L-cells that did not aggregate with transfected cells remained single (Fig. 6, small arrows) or formed pairs and triplets in a similar rate as in the control mixture, whereas transfected cells were frequently observed to associate only with other transfected cells. Morphologically, the heterotypic aggregates appeared to consist of cores of green fluorescent cells, comprising over 90% of the counted cells expressing either Claudin-1 or Caecn2, with PKH26-labeled L-cells located on the outside. Individual Claudin-1 or Caecn2 expressing cells that did not aggregate were rare (Fig. 6, large arrowheads). These data indicate that both Claudin-1 and Caecn2 are able to bind in a heterotypic manner.

**Aggregated Caecn2 Expressing Cells Exhibit Close Intermembrane Contact**—Cell-cell adhesions formed by Claudin-1 transfected L-cells are characterized by very close apposition of the plasma membranes and the appearance of kissing points under ultrathin section electron microscopy (41). To extend our evaluation of the functional similarity of Caecn2 to the claudin protein family, we performed electron microscopy (EM) on sections taken through control L-cells, and L-cells expressing either Claudin-1 or Caecn2, following 4 h of aggregation. Because untransfected L-cells do not aggregate well, it was difficult to identify many instances where these cells remained in proximity following fixation and embedding but, where these were observed under EM, the plasma membranes themselves remained separated by relatively large gaps that were often occupied by filamntous cellular processes (Fig. 7, top). Claudin-1 and Caecn2 expressing cells, on the other hand, formed aggregates with close contact between the plasma membranes of adhering cells (Fig. 7, bottom). These Claudin-1- and Caecn2-mediated contacts appeared indistinguishable from each other.

**DISCUSSION**

Claudins are cell adhesion molecules that selectively regulate paracellular permeability at epithelial tight junctions (32, 33). The related Ca\(^{2+}\) channel \(\gamma\) subunit proteins are primarily expressed in neurons, where they function as regulators of voltage-gated ion channels and neurotransmitter-gated ion channels (9, 10, 26, 27, 45, 46). Based on the strong evolution-
expressed in mouse L-fibroblast cells, Claudin-1 and Caeng2 (stargazin) both localized to the plasma membrane and were capable of inducing cell aggregation. These results are consistent with the idea that cell-cell adhesion is an ancient function of the claudin and Ca\(^{2+}\) channel \(\gamma\) subunit proteins, and that the adaptive expansion of these gene families from a common ancestor occurred without compromising this basic ability.

In our study, we initially employed methods similar to those originally used to demonstrate that Claudin-1, -2, and -3 induced a rapid and Ca\(^{2+}\) independent aggregation of L-cells (41). With this technique, cells are suspended in HCMF (HEPES-buffered saline minus Ca\(^{2+}\) and Mg\(^{2+}\) plus 1 mM EDTA) and allowed to aggregate during rotation at 80 rpm. However, we observed sporadic cell damage during rotary shaking in this buffer that resulted in unacceptable levels of nonspecific aggregation, possibly because of the liberation of sticky genomic DNA from lysed cells. For this reason, we switched to an alternate protocol based on standard culture medium (Dubcco’s modified Eagle’s medium \(\pm\) EGTA) that had been used to study cadherin-mediated aggregation in L-cells (37). Using this assay, the aggregation of Claudin-1 expressing cells occurred at between 1 and 4 h, which is significantly slower than the \(<\sim\)30 min reported for Claudin-1 cells in HCMF buffer (41). We were also surprised to discover that the aggregation mediated by Claudin-1 was Ca\(^{2+}\) dependent.

The precise reasons for the differences in Ca\(^{2+}\) dependence and time course of Claudin-1 aggregation in these studies are unclear. However, it is well known that adhesion at some types of cell-cell contacts, including adherens junctions and TJs, is Ca\(^{2+}\) dependent, and that Ca\(^{2+}\) chelation leads to loss of adhesion and the internalization of proteins at these structures (47). Recent analyses of Claudin-1 expression in MDCK cells are consistent with our observations. For example, Kobayashi et al. (48) observed that in MDCK cells cultured for 24 h in low Ca\(^{2+}\) (<5 \(\mu\)M) medium, endogenous Claudin-1 was no longer visible at the plasma membrane but had become distributed within the cytoplasm in a punctate pattern. One hour after switching to normal Ca\(^{2+}\) (1.8 mM) medium, Claudin-1 had translocated back to the plasma membrane at points of cell-cell contact. Rothen-Rutishauser et al. (49) used the Ca\(^{2+}\) chelation method to show that Claudin-1 was rapidly internalized in MDCK cells, from the plasma membrane to cytoplasmic bodies, after only 20 min of treatment with 2 mM EGTA. Sixty minutes following the restoration of normal Ca\(^{2+}\) levels, Claudin-1 immunoreactivity began to reappear at the plasma membrane and, 5 h later, was no longer visibly concentrated in the cytoplasm. There are significant differences between MDCK cells and L-fibroblasts, including that MDCK cells form monolayers with well-developed TJs in culture, whereas L-cells do not, but these studies demonstrate that Claudin-1 subcellular localization, whether directly or secondarily through other molecules, can be exquisitely sensitive to alterations in Ca\(^{2+}\) concentration in cells assayed in standard culture medium. It is possible that the analysis of Claudin-1 aggregation in HCMF buffer (41) does not reproduce the Ca\(^{2+}\) dependence observed in the supplemented Dubcco’s modified Eagle’s medium used in our assay or the MDCK cell studies described above, for reasons intrinsic to the composition of these solutions that are unrelated to Ca\(^{2+}\). For example, it may be significant that we used EGTA as the Ca\(^{2+}\) chelator in our analysis, which binds Ca\(^{2+}\) with considerably greater affinity than other divalent cations (e.g. Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\)), whereas Kubota et al. (41) used EDTA, which is less specific for Ca\(^{2+}\). These other cations have been shown to regulate TJ integrity through mechanisms that are not well understood (50).
One of the most conspicuous differences between claudins and Ca\textsuperscript{2+} channel \(\gamma\) subunits is that the latter each display at least one consensus motif for \(N\)-linked glycosylation, whereas only 2 of the 20 claudin proteins we compared (CLDN1 and CLDN12) possess this site. Cacng2 localizes to neuronal synapses (13, 27) and there is growing evidence that carbohydrates play a unique and important role in regulating synaptic function (51, 52). Therefore, we sought to explore the functional relevance of \(\gamma\) subunit glycosylation by generating a substitution mutation (glutamine for asparagine) in the sole consensus \(N\)-glycosylation site of Cacng2. Expression of Cacng2-N48Q was confirmed by immunoblotting (Fig. 2) but this protein was not correctly targeted to the plasma membrane (Fig. 4) and did not induce cells to aggregate (Fig. 3, I and J). \(N\)-Glycosylation is known to be critical for the correct folding, subcellular targeting, and stability of numerous proteins (53) so this result was not surprising. Nonetheless, it is interesting to consider that \(N\)-glycosylation might have evolved to become essential for the normal post-translational processing or trafficking of Cacng2, and possibly the other \(\gamma\) subunit proteins, but not for claudins, which appear to share the same basic structure. Of course, it is also possible that the conversion of asparagine to glutamine had a deleterious effect on Cacng2 folding or trafficking that had little to do with the prevention of glycosylation at this site. Supporting this possibility, several mutations in the related PMP-22 and LIM-2 proteins that do not disrupt glycosylation sites are characterized by loss of function because of retention in the endoplasmic reticulum or other subcellular compartments (54, 55). Experiments utilizing a variety of glycosylation inhibitors and deglycosylating enzymes will be useful for addressing this issue in the future.

Interactions between cell adhesion proteins may be broadly classified as either homotypic (between proteins of the same type) or heterotypic (between different proteins). We presented

**Fig. 6.** Heterotypic adhesion of untransfected fibroblast cells to aggregates of Claudin-1 or Cacng2-expressing cells. PKH26-labeled untransfected L-cells (red) were mixed 1:1 with unlabeled untransfected L-cells (top row), GFP-Claudin-1-expressing L-cells (middle row), or GFP-Cacng2-expressing L-cells (bottom row). The mixtures were allowed to aggregate during 4 h of rotary shaking (80 rpm at 37 °C). Matching fields were photographed with differential interference contrast (DIC) optics, or with rhodamine (PKH26 red) or fluorescein (GFP) filters. The top row shows that PKH26-labeled or unlabeled untransfected L-cells do not adhere to one another. The middle and bottom rows indicate that the majority (~80%) of untransfected L-cells were contained in aggregates. When unassociated cells were observed, they were usually found to be untransfected L-cells (arrows). A rare, unaggregated Cacng2-expressing cell is indicated by an arrowhead in the bottom panel. Scale bar = 100 μm.

**Fig. 7.** Aggregating Cacng2 expressing cells exhibit close intermembraneous contact. Electron microscopy of sections through adjacent control L-cells shows that they are non-adherent, with long filamentous cellular processes intervening between them (top; scale bar = 2 μm). In contrast, Claudin-1 and Cacng2 expressing cells formed aggregates with apposing membranes in close proximity. Higher magnification views demonstrate typical adhesions between Claudin-1 and Cacng2-expressing cells, with kissing points (arrows) as well as regions where individual membranes are indistinct (see the left edge in Cldn-1 and the region between the two right-hand arrows in Cacng2). Scale bar = 200 nm.
evidence suggesting that the adhesion mediated by Claudin-1 or CaCng2 is heterotypic (Fig. 6), but this does not rule out the possibility that these molecules can also bind homotypically. For example, it was previously shown that Claudin-1, -2, and -3 could bind homotypically, whereas heterotypic interactions were also possible between Claudin-1 and -3 and Claudin-2 and -3, but not between Claudin-1 and -2 (56). Untransfected L-cells do not express CaCng2 (Fig. 2) or Claudin-1, -2, or -3 (41), so it is not obvious how they are able to join in aggregates with CaCng2 or Claudin-1 expressing cells. These results suggest that L-cells express proteins, perhaps other members of the claudin superfamily, which have the ability to interact heterotypically with either CaCng2 or Claudin-1, but which cannot interact homotypically among themselves. In this respect, it is notable that CaCng2 appears to be predominantly localized to postsynaptic neuronal membranes in vivo, but has not been detected in presynaptic membranes or in glia (6, 23, 27). Thus, if CaCng2 does mediate adhesion between neurons, or between neurons and other cell types, it is likely to be through a heterotypic interaction. Investigations into the ability of the other seven Ca2\(^{2+}\) channel \(\gamma\) subunit proteins (CaCng1 and CaCng3–8) to function as homo- or heterotypic cell adhesion molecules are currently underway.

Our use of L-fibroblasts to test whether CaCng2 could mediate cell adhesion was necessitated by the fact that most cell types, particularly neurons, possess strong endogenous cell adhesion capabilities that preclude their use in classical cell aggregation assays. However, CaCng2 is expressed exclusively in neurons in vivo and, regardless of whether or not CaCng2 functions like Claudin-1 in L-cells, neurons do not form TJs. So, what role might CaCng2-mediated cell adhesion play in neurons? CaCng2 and Claudin-1 proteins diverged from a common ancestor. The analogous possibility exists that there are cell adhesion structures in modern neurons that share an ancient morphological ancestor with modern tight junctions. Identifying such relationships might provide useful clues to CaCng2 function. In this regard, it is interesting that neurons and epithelial cells share a common developmental origin in embryonic ectoderm, and that morphologically, both are highly polarized cell types. Furthermore, recent investigations have demonstrated that epithelial TJs, like neuronal synapses, are important sites for the localization of molecules involved in vesicle docking/targeting, signaling, and orienting polarized membrane traffic, including: syntaxins, synaptosome-associated proteins, synaptotobrevin/vesicle-associated membrane proteins, and PDZ domain proteins (57).

Neurons exhibit several types of specialized cell-cell contacts, including classical synapses, puncta adherens junctions, reticular adherent junctions, gap junctions, and paranodal axoglial junctions (58, 59). CaCng2 could be involved in intermembrane interactions at one or more of these structures, particularly at synapses and puncta adherens junctions whose sites are most consistent with the location of CaCng2 at or near postsynaptic densities. Are there any structural abnormalities in stargazer mouse neurons that might be consistent with a cell adhesion defect in these structures? CaCng2 is highly expressed in cerebellum (6, 23) and, because CaCng2-dependent AMPA receptor defects are severe in cerebellar granule cells (10), most studies of stargazer brain have focused on this region. Despite a mild (~14%) decrease in the gross wet weight of stargazer cerebellum relative to wild type, no obvious cytoarchitectural abnormalities in foliation or laminar structure have been described (60, 61). Furthermore, qualitative ultrastructural examination of excitatory mossy fiber-granule cell and parallel fiber-Purkinje cell synapses by electron microscopy revealed grossly normal synaptic structures in stargazer cerebellum (61, 62). The results of recent quantitative analyses are more intriguing. Experiments using immunogold labeling electron microscopy revealed decreases in the length of the active zone at parallel fiber-Purkinje cell synapses, reduced thickness of the postsynaptic density at mossy fiber-granule cell, and parallel fiber-Purkinje cell synapses, and decreases in the number of docked neurotransmitter vesicles in parallel fiber terminals in stargazer relative to wild type mice (62, 63). Although a causal relationship between defective cell adhesion and these types of ultrastructural abnormalities has not been shown, it is worth noting that other cell adhesion molecules, including those located within puncta adherens junctions, have been implicated in activity-dependent synaptogenesis and synaptic plasticity (64, 65). The absence of any obvious evidence of decreased cell adhesion between neurons, or between neurons and glia, in stargazer brain may simply reflect the fact that these cells express several other strong cell adhesion proteins, including N-cadherin, NCAM, L1CAM, SynCAM, neuroligin/\(\beta\)-neurexin, and the \(\gamma\)-protocadherins (66), whose activity might effectively mask the loss of any cell adhesion that might have been conferred by CaCng2.

There is now considerable evidence that Ca2\(^{2+}\) channel \(\gamma\) subunits function as regulators of voltage- and neurotransmitter-gated ion channels, so it seems reasonable to ask how these neuronal signaling complexes could benefit by evolving an interaction with a cell adhesion molecule. Important clues may be obtained from recent studies of another type of ion channel regulatory protein, the voltage-gated Na\(^+\) channel \(\beta\) subunit, which has also been shown to function as a cell adhesion molecule. Na\(^{+}\) channel \(\beta_1\) and \(\beta_2\) subunits are immunoglobulin-related cell adhesion molecules that regulate channel expression at the plasma membrane and at axonal nodes of Ranvier, modulate channel gating, control interactions with the cytoskeleton, and promote neurite outgrowth (67–69). CaCng2 might act in a similar fashion, as a subunit of neuronal voltage-gated Ca2\(^{2+}\) channels or AMPA-type glutamate receptors. Mutations in the mouse and human \(\beta_1\) subunit genes (Scn1b, SCN1B) were recently shown to cause epilepsy (70, 71). CaCng2, mutated in the stargazer mouse, thus represents the second ion channel-regulating cell adhesion molecule to be associated with this phenotype. The possibility that CaCng2 and the other neuronal \(\gamma\) subunit proteins function to colocalize and align voltage- and neurotransmitter-gated ion channels at sites of close membrane apposition between two cells, to maximize the efficiency of intercellular communication in the brain, will be an exciting topic for future investigation.

Acknowledgments—We thank Signe Carlson and Shelu Patel for technical assistance and Dr. Jeffrey L. Noebels, Dr. Kristen Senechal, Dr. Malcolm S. Steinberg, and Carolyn Zilinski for valuable advice.

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The \( \alpha \)-Amino-3-hydroxyl-5-methyl-4-isoxazolepropionate Receptor Trafficking Regulator "Stargazin" Is Related to the Claudin Family of Proteins by Its Ability to Mediate Cell-Cell Adhesion

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*J. Biol. Chem.* 2005, 280:19711-19720.

doi: 10.1074/jbc.M500623200 originally published online March 10, 2005