γ-Protocadherins (γ-pcdhs) are type I membrane-spanning glycoproteins, widely expressed in the mammal and required for survival. These cell adhesion molecules are expressed from a complex locus comprising 22 functional variable exons arranged in tandem, each encoding extracellular, transmembrane and intracellular sequence, and three exons for an invariant C-terminal domain (γ-ICD). However, the signaling mechanisms that lie downstream of γ-pcdhs have not been elucidated. Here we report that γ-pcdhs are subject to presenilin-dependent intramembrane cleavage (PS-IP), accompanied by shedding of the extracellular domain. The cleaved intracellular domain (γ-ICD) translocates to the cell nucleus and was detected in subsets of cortical neurons. Notably, gene-targeted mice lacking functional γ-ICD sequence showed severely reduced γ-pcdh mRNA levels and neonatal lethality. Most importantly, inhibition of γ-secretase decreased γ-pcdh locus expression. Luciferase reporter assays demonstrated that γ-pcdh promoter activity is increased by γ-ICD. These results reveal an intracellular signaling mechanism for γ-pcdhs and identify a novel vital target for the γ-secretase complex.

Cadherins represent a large superfamily of transmembrane glycoproteins, sharing common structural features, but exhibiting differential adhesive binding specificities (1). Characterized best are the so-called classic cadherins, including E-, R-, N-, and P-cadherin, and the remainder forms subgroupings collectively referred to as protocadherins (pcdhs). Classic cadherins are characterized by an ectodomain consisting of 5 extracellular cadherin-like (EC) repeats, followed by a single transmembrane domain and a highly conserved C-terminal cytoplasmic region. Classic cadherins engage mostly in homophilic adhesive binding triggered by Ca²⁺ ions intercalating between the EC domains to produce a rigidified, rod-like ectodomain (2). Intracellularly, classic cadherins associate with different proteins, including catenins, that regulate their adhesive properties or mediate downstream signaling (3), which is important for establishing brain structure and connectivity including synaptic structure, function and plasticity (4–9). Besides well documented signaling function via catenins, E- and N-cadherin have been shown to undergo presenilin dependent intramembrane proteolysis (PS-IP), which consists of matrix protease-mediated cleavage of the ectodomain and the characteristic release of a soluble cytoplasmic domain by the activity of the γ-secretase complex and its catalytic constituents presenilin-1/2 (10, 11). The released cytoplasmic cadherin domains can assume signaling function in the cytoplasm and/or nucleus, in analogy to the cytoplasmic domains of prominent targets of γ-secretase, Notch, APP, ErbB-4, and SREBP-1 (12–15).

In comparison to classic cadherins, pcdhs differ in the number of EC domains and have divergent intracellular domains. Recently, >50 novel type-I transmembrane pcdhs have been described (16, 17), of which most, if not all, are expressed in brain. They are thought to participate in specific synaptic connections based on their structural similarities to cell adhesion molecules and their synaptic localization (18–21). As a characteristic feature these pcdhs harbor six EC domains related to those of classic cadherins, but contain different cytoplasmic domains. In human and mouse they are arranged in three physically linked clusters, termed α, β, and γ, located on mouse chromosome 18 (human chromosome 5). Each cluster contains multiple tandemly arranged functional variable exons (14 for α, 22 each for β and γ), each encoding the extracellular sequence, the transmembrane region and part of the intracellular domain. Transcription of each α- and γ-pcdh variable exon is initiated at sequences immediately upstream of the translational start site (22, 23). Notably, each variable γ-pcdh exon contains its own promoter/enhancer, including a conserved sequence element (CSE) essential for proper transcription, ~200-bp upstream of the translational start site (22). In addition to the variable exon array, the α- and γ-pcdh, but not the β-pcdh, locus harbor three exons 3' of the variable exon cluster, which together encode invariant cytoplasmic domains (α-ICD, γ-ICD) (16, 17). Thus, cis-splicing of the cluster transcripts produces α- and γ-pcdh isoforms with distinct adhesive properties but identical cluster specific intracellular domains.

The γ-pcdh locus represents the best studied of the three subclusters of pcdhs. Initial investigations revealed that the γ-pcdh genes are abundantly transcribed in neurons. Importantly, an intact γ-pcdh locus is required, as demonstrated by neonatal death of mice from locus deletion (19). Global aspects of neurogenesis, including neuronal migration, axonal outgrowth and synapse formation appeared unaffected, but specific neuronal populations, especially spinal interneurons, died at late stages of development, indicating a critical requirement for γ-pcdh-mediated signaling for their survival.

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As yet, the mode and mechanism of γ-pcdh-mediated signal-
ning are unknown. An intriguing aspect is certainly provided by the
γ-ICD, which as integral part of all 22 functionally ex-
pressed γ-pcdhs is likely to interact and provide cross-talk with
cellular signaling pathways. However, no proteins interacting with
the γ-ICD have been described. Here we provide evidence that
the γ-ICD can be released by PS-IP from γ-pcdhs inserted in the
plasma membrane and translocates to the nucleus where the
γ-pcdh locus appears to be a potential target for γ-ICD-
mediated transcriptional enhancement by as yet unknown mecha-
nisms. This autoregulatory aspect of γ-pcdh expression may contribute to unexpectedly low γ-pcdh levels in mice het-
erygous for a γ-pcdh locus lacking γ-ICD exon sequence.

EXPERIMENTAL PROCEDURES

Generation of Mice with Flxed γ-pcdh Alleles—The γ-pcdh targeting construct was generated from a ~15-kb 129/Sv mouse genomic DNA
isolated from a Stratagene λ Fix2 library with radiolabeled γC1 sequence. A ~12-kb XhoI/BstBI fragment was subcloned and a floxed
pgk-neo cassette from vector ploxP-neo (23) was inserted into the unique SacII site of vector pGL3-E (Promega). We used: for
- GCTCTAGAACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGCAAGCC-
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- GACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGCAAGCC-
and GX2, 5′-GGGGTACCGTCTGTCTTCATTTCTATTT-
- CAGGACATAGTGGATTGGCCCC-3′ and GX2, 5′-GCTCTAGACTAGCCAAGGGCTCTCTAGT-
- ICDD-10his a
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Western blots, Immunofluorescence, and Co-immunoprecipitations—For Western blot (WB) analysis cultured cells or mouse brains were solubilized in 25 mM HEPES, pH 7.5, 150 mM NaCl, protease inhibitor mixture (Roche Applied Science), containing 1% Triton X-100. SDS-PAGE and blotting were performed using standard conditions. γ-ICD rabbit polyclonal antiserum was used in 1:200 (fixed cells) or 1:500 (WB) dilutions, all other antisera were diluted according to manufacturer’s protocols. Immunofluorescence was performed using standard protocols. To visualize nuclei, cells were treated with 4′,6-diamidino-2-phenylindole (DAPI, 1:5000; Sigma) for 5 min at room temperature. Mounted sections were examined in a Zeiss fluorescence microscope. Quantification of Western blots was performed with ImageJ software. For co-immunoprecipitation COS-1 cells were cotransfected with vectors for Flag-pcdh-B1 and KVP-pcdh-A1 or GFP-pcdh-B1 or KVP-pcdh-A1ΔCT or KVP-pcdh-A1ΔICD. All subsequent operations were at 4°C. Cell lysates were preclued with 25 μl of immobilized protein A (ImmunPure®, Pierce) for 30 min, and ~400 μl of (protein concentration ~1 μg/μl) were incubated with ~5 μg of antibody (AFP or Myc) overnight. Precipitates were recovered after 4 h of incubation with 25 μl of bead-immobilized protein A. Beads were washed 4× with 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and bound material was eluted at 99°C in 20 μl of 3× sample buffer.

Real Time PCR—An ABIPRISM 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA) was used for cDNA quantification. Total RNA was isolated from HEK293 cells cotransfected with vectors for GFP and KVP-pcdh-A1 or KVP-pcdh-A1ΔCT or KVP-pcdh-A1ΔICD or from pharmacologically treated SH-SY5Y cells. RNAs were DNase I-treated and reverse-transcribed. About 1–2 μg of the resulting cDNA was analyzed in a reaction volume of 25 μl (on 96-well plates), using the TaqMan PCR Core reagent kit (PE Applied Biosystems). cDNA of each cell culture dish was determined in a Beckman luminometer. Cotransfections were performed in 6-well plates with for Flag-pcdh-B1 and KVP-pcdh-A1 or GFP-pcdh-B1 or KVP-pcdh-A1ΔCT or KVP-pcdh-A1ΔICD. All subsequent operations were at 4°C. Cell lysates were preclued with 25 μl of immobilized protein A (ImmunPure®, Pierce) for 30 min, and ~400 μl of (protein concentration ~1 μg/μl) were incubated with ~5 μg of antibody (AFP or Myc) overnight. Precipitates were recovered after 4 h of incubation with 25 μl of bead-immobilized protein A. Beads were washed 4× with 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and bound material was eluted at 99°C in 20 μl of 3× sample buffer.

Results—Attenuated γ-pcdh Locus Expression and Neonatal Lethality in Mice Lacking the Invariant γ-pcdh C-terminal Domain—The mouse γ-pcdh locus (Fig. 1A) was targeted in murine embryonic stem cells (25) to generate a γ-pcdhneo allele (neo) in which γ-C1, the first of the three exons, γ-C1 to γ-C3, that encode the γ-ICD (Fig. 1B), was floxed, by introducing a loxP site in the intron upstream of γ-C1 and a floxed pgtk-neo gene into the downstream intron. The neo allele became precursor to the γ-pcdhΔC1 allele (ΔC1) with γ-C1 deleted, produced by crossing +/neo mice with a Cre deleter strain (26). Removal of γ-C1 generates a frameshift (Fig. 1B) and hence, the ΔC1 allele cannot express functional γ-ICD.

Heterozygous +/-neo and +/-ΔC1 mice appeared phenotypically inconspicuous and generated upon intracrossing homozygous neo/neo and ΔC1/ΔC1 offspring at Mendelian frequency (mice wild type, heterozygous, homozygous: neo 21, 34, 13; ΔC1 12, 24, 11). Notably, all homozygous pups died during the first 12 h after birth, as described for newborn mice lacking the entire γ-pcdh locus (19). Hence, to evaluate splicing and expression of transcripts from the altered γ-pcdh alleles, we subjected brain RNA from newborns of different genotypes to RT-PCR and Northern analyses.

Splicing from variable to constant region exons was determined by RT-PCR with sense oligonucleotide primers generic for variable region exons A1–12, or specific sense primers for variable exons A8 (not shown) and B4, in combination with an antisense primer within γ-C3. The amplicons, resolved on agarose gels (Fig. 1C) and checked by DNA sequencing (not shown), revealed that the pgtk-neo insertion did not cause skipping of γ-C1 or γ-C2, and that loss of γ-C1 resulted in splicing from variable exons to γ-C2, with no detectable band corresponding to a splice directly to γ-C3. Moreover, the skewed ratio of the two RT-PCR fragments, respectively containing or lacking γ-C1 exon sequence, from RNA of heterozygous +/-ΔC1 mice (Fig. 1C) documented that spliced transcripts from the ΔC1 allele were in much lower abundance than those from the wild-type γ-pcdh allele.

Quantification by Northern analysis from poly(A)+ RNA with four probes for variable and conserved exons showed that the amount of the ~4.4-kb γ-pcdh mRNA was in +/-neo mice little over, and in +/-ΔC1 significantly below, the 50% expected from the intact wild-type allele present in these genotypes (Fig. 2A and B). This indicated that both modified alleles (neo, ΔC1) were severely impaired in mRNA production. Moreover, the analysis revealed that the wild-type γ-pcdh allele in +/-ΔC1 mice produced subnormal mRNA amounts. In neo mice, γ-pcdh mRNA was reduced to ~20% of wild-type levels; the intronic pgtk-neo insertion may have resulted in reduced splicing efficacy of the allelic neo transcripts. In ΔC1/ΔC1 mice, γ-pcdh mRNA lacking γ-C1 sequence was <10% of wild-type levels.

We also evaluated γ-pcdh protein levels in the different genotypes. The γ-pcdh specific ~114 kDa protein band reacting on Western blots with our antibody showed relative to wild type an intensity of ~60% in +/-neo mice, and ~10% in neo/neo mice (Fig. 2, C and D), in good correspondence to the respective mRNA levels. Thus, low γ-pcdh levels might underlie the perinatal death of neo/neo mice. Most notably, the Western analysis of brain lysates from +/-ΔC1 mice revealed steady-state γ-pcdh levels of ~25% of wild type, even though twice as much should be generated by the remaining functional wild-type allele alone (Fig. 2, C and D). Apparently, such low γ-pcdh levels suffice for the survival. We finally evaluated possible effects of the modified γ-pcdh alleles on mRNA levels of the physiologically proximal, paralogous α-pcdh locus, but found none (data not shown).

N- and C-terminal γ-pcdh Portions Localize Differentially in Cells—In situ hybridization with an oligonucleotide probe to γ-C2 on coronal brain sections of adult wild-type mice and on
saggital sections of embryonic day 14 embryos documented prominent neuronal γ-pcdh transcript distribution in CNS (Fig. 3A). Staining of forebrain sections of adult mice from different brain regions with our anti-γ-ICD antibody revealed that subsets of neurons showed strong immunoreactivity, with most neurons having stained nuclei (Fig. 3B, inset). The nuclear staining was not apparent in sections from newborn mice (Fig. 3C, upper panel). The specificity of staining was demonstrated by competition with excess γ-ICD, omitting of the primary antibody (Fig. 3B, right panel, inset), and in AC1/AC1 newborns lacking γ-ICD (Fig. 3C, lower panel).

To trace the cellular distribution of the γ-ICD in a system amenable to pharmacological intervention, we constructed a vector for N-terminal fusion of EYFP to γ-pcdh-A1 (EYFP-γ-pcdh-A1) and expressed the fusion protein transiently in COS-1 cells. Anti-GFP antibody staining on nonpermeabilized cells showed fluorescent protein clusters on the cell surface, clearly indicating that ectopically expressed EYFP-γ-pcdh-A1 was efficiently inserted into the plasma membrane (Fig. 3D, panel 1). Direct EYFP-mediated fluorescence was exclusively detected in the cytoplasm, with little or no fluorescent signal visible in the nucleus (Fig. 3D, panel 2), showing that the N-terminal portion of EYFP-γ-pcdh-A1 is absent in the nuclear compartment. However, antibody staining against the γ-ICD, after permeabilizing transfected COS-1 cells and use of a fluorescein isothiocyanate-labeled secondary antibody, revealed strong fluorescence in both, nucleus and cytoplasm (Fig. 3D, panel 3), as further confirmed by counterstaining with DAPI (not shown). This concurs with the endogenous γ-ICD signal in mouse brain sections.

Detecting N- and C-terminal portions of γ-pcdhs in different subcellular compartments could reflect proteolytic processing of γ-pcdhs and nuclear translocation of γ-ICD, which is predicted to contain a bipartite nuclear localization sequence (Fig. 1B). To test if γ-ICD gets into the nucleus, we expressed it as Myc-fusion (Myc-γ-ICD) ectopically in COS-1 cells. Indeed, recruitment of Myc-ICD into the nucleus was evinced by the strong nuclear signal generated by both anti-Myc and anti-γ-ICD antibodies (Fig. 3D, panels 4 and 5). Moreover, staining of untransfected cells, photographed with longer exposure time, documented nuclear localization also of the endogenous γ-ICD (Fig. 3D, panel 6).

γ-pcdhs Undergo Intramembrane Proteolysis by γ-Secretase—A likely candidate for proteolytic processing of γ-pcdhs leading to intracellular release of γ-ICD sequence is the γ-secretase complex, which has recently been implicated in the processing of a growing number of membrane proteins (35–37). To test if γ-secretase is involved in γ-pcdh processing, we treated COS-1 cells transiently expressing EYFP-γ-pcdh-A1 with specific γ-secretase inhibitors (L-685,458 and DAPT) and monitored the accumulation of a cytoplasmic membrane-bound cleavage intermediate, which should appear following ectodomain shedding and γ-secretase inhibition (Fig. 4A). Both substances efficiently inhibited cleavage of transiently transfected APP2Z (38), a well-described target of the γ-secretase complex (Supplementary Fig. 1). In case of transiently expressed EYFP-γ-
pcdh-A1, Western analysis with our antibody against the γ-ICD showed significant accumulation of a ~26 kDa (γ-26 kDa) band by inhibition of γ-secretase (Fig. 4 A). Moreover, an antibody-reactive ~20 kDa (γ-20 kDa) fragment, consistently present at lower levels than the γ-26 kDa band, was conspicuously absent in cells treated with the γ-secretase inhibitors. This fragment is of the expected size for the intracellular pcdh portion released by γ-secretase (Fig. 4 A). When transfected cells were treated for 24 h with brefeldin A, a compound disassembling the Golgi apparatus, or monensin, which prevents transfer of proteins from Golgi to plasma membrane, no cleavage products of EYFPγ-pcdh-A1 were detected in Western blots (Supplementary Fig. 1), indicating that γ-pcdh insertion into the cell membrane is mandatory for processing by γ-secretase.

As γ-secretase requires prior extracellular protease cleavage, resulting in shedding the substrate’s ectodomain, we collected the medium of EYFPγ-pcdh-A1 expressing COS-1 cells and probed Western-blotted medium protein with antibody against the N-terminal EYFP moiety of the fusion protein. This analysis revealed two shedded cleavage products, with the larger one efficiently blocked by TAPI, a matrix metalloprotease inhibitor. A different enzyme may release the smaller shedded product. In any event, the significant decrease in the amount of the γ-26 kDa and γ-20 kDa fragments from lysates of TAPI-treated relative to untreated cells (Fig. 4 A) demonstrated the influence of matrix metalloprotease cleavage on γ-secretase efficacy.

The catalytic components of the γ-secretase complex are the presenilins (39–41). To support the pharmacological evidence for γ-pcdh processing by γ-secretase, we transiently transfected presenilin-1/2 double knockout (dko) cells (33, 34) with vector for EYFPγ-pcdh-A1. Subsequent Western analysis with our antibody for γ-ICD revealed accumulation of the presumptive membrane-bound γ-26 kDa fragment and absence of the γ-20-kDa fragment.

**Fig. 2. Expression of γ-pcdh alleles with altered constant region.** A, Northern blot analyses of poly(A)+ RNA from neonatal brains of the different genotypes. Blots were hybridized with a generic variable region probe (γ-VAR) and probes for γ-C1, γ-C2, and γ-C2C3. RNA quantities were evaluated with a cyclophilin probe (CyP). B, quantification of relative transcript abundance from Northern blots. Relative signal intensities are plotted versus genotype, with values for wild type set at 100%. Shown are averaged results (mean ± S.D.) from five mice for each genotype from at least three independent experiments. C, Western blot analysis of protein lysates from neonatal brains of the different genotypes. The anti-γ-ICD antibody detected the γ-pcdh specific band. Asterisks indicate unspecific bands detected by the antibody. D, quantification of relative protein amounts from Western blots. Relative signal intensities are plotted versus genotype, with values for wild type set at 100% (mean ± S.D.). Shown are averaged results from five mice for each genotype from at least three independent experiments. Note that the low protein levels detected in +/ΔC1 mice are in congruence with the quantification of the transcript levels by Northern blots.
In accordance, the subcellular distribution of the γ-ICD was strikingly affected. EYFP-γ-pcdh-A1 expressing dko cells, unlike COS-1 cells, showed γ-ICD specific signal exclusively in cytoplasm (Fig. 4B, left and middle panels). However, dko cells transiently expressing γICD exhibited intense nuclear localization of this protein, demonstrating that nuclear import was not impaired in these cells (Fig. 4B, right panel). Similar results were obtained for GFP-γ-pcdh-B1 (data not shown).

Collectively, these data indicate that γ-pcdhs in the plasma membrane are initially cleaved by a TAPI-sensitive matrix metalloprotease and presumably by other matrix proteases, leading to shedding of the ectodomain, and are additionally processed by the γ-secretase complex to release a soluble cytoplasmic fragment containing γ-ICD sequence, which translocates to the nucleus. These events are defining features of PS-IP.
**Fig. 4. Intramembrane proteolysis of γ-pcdhs.** A, upper panel, Western analysis of medium collected from EYFP-γ-pcdh-A1 expressing COS-1 and dko cells incubated with TAPI, DAPT or L-685,458 and probed with anti-GFP antibody. Black arrowheads point to two shedded ectodomain products (γ-EC (1–6)) indicating two matrix protease cleavages (lanes 2–5). Loss of the larger fragment by TAPI reveals the participation of a matrix metalloprotease in ectodomain shedding (lane 1). Lower panel, Western analysis of corresponding cell extracts with our anti-γ-ICD antibody (lanes 1–5). Black arrowheads point at specific cleavage products. DAPT and L-685,458 treatment leads to enrichment of the γ-26-kDa fragment and loss of the γ-20-kDa fragment. Note the absence of the γ-20-kDa fragment in dko cells (lane 5). A schematic illustration of proposed events in EYFP-γ-pcdh-A1 processing is shown next to the Western blots. The γ-26-kDa fragment is produced by matrix protease cleavage (MP), and the γ-20 kDa fragment by γ-secretase (γ-secretase, open arrow). The γ-ICD corresponds to the filled rectangle. B, left panel, EYFP-γ-pcdh-A1 expressing permeabilized dko cells show fluorescence exclusively in the cytoplasm upon staining with anti-γ-ICD antibody. Middle panel, direct fluorescence of EYFP-γ-pcdh-A1 in the cytoplasm of transfected dko cells. Right panel, mγ-γ-ICD-transfected dko cells show intensive staining in the nucleus after anti-γ-ICD staining.

**γ-ICD Promotes γ-pcdh Locus Expression**—Nuclear import of γ-ICD following processing of γ-pcdhs by PS-IP suggests that this invariant protein domain may be involved in regulating transcription, in analogy to the Notch paradigm (12). We surmised that target genes might include the γ-pcdh locus, given that overexpressing in HEK293 cells myc-γ-ICD but not mγ-γ-ICD, the structurally unrelated invariant C-terminal domain of α-pcdhs (16, 18), significantly increased γ-pcdh levels (Fig. 5A, left panel).

Curiously, cells overexpressing EYFP-γ-pcdh-A1ΔCT, a protein without the γ-ICD, also exhibited an increased endogenous γ-pcdh signal when compared with cells overexpressing empty vector, mγ-γ-ICD (Fig. 5A, left panel) or CD-8 protein (not shown). We assume that EYFP-γ-pcdh-A1ΔCT stabilizes endogenously expressed γ-pcdhs, perhaps by heterodimer formation, as demonstrated by co-immunoprecipitations (Supplementary Fig. 1). In support, real-time PCR quantification from transfected HEK293 cells revealed a 4-fold up-regulation of endogenous γ-pcdh transcripts in samples overexpressing mγ-γ-ICD but not EYFP-γ-pcdh-A1ΔCT (Fig. 5A, right panel). Likewise, recombinant mγ-γ-ICD expression did not significantly alter endogenous γ-pcdh transcript levels. Thus, an overexpressed γ-pcdh variant stabilizes endogenous γ-pcdhs without affecting γ-pcdh locus transcription, but overexpressed γ-ICD enhances endogenous γ-pcdh transcription.

We further investigated the effect of γ-secretase inhibition on γ-pcdh locus expression. Human SH-SY5Y cells, which responded to pharmacological intervention better than HEK293 cells, were exposed to the γ-secretase inhibitor DAPT and compared in γ-pcdh expression to mock (Me2SO)-treated cells. Protein lysates showed a reduction in endogenous γ-pcdh levels of ~35% (Fig. 5B, left panel). Real-time PCR quantification indicated a similar reduction in endogenous γ-pcdh transcripts after inhibiting γ-secretase (Fig. 5B, right panel). We conclude that γ-pcdh locus expression is responsive to γ-secretase-mediated release of the γ-ICD.

**Transactivation of γ-pcdh Promoters by γ-ICD**—Our results suggested that the γ-ICD enhances γ-pcdh gene locus expression. We therefore tested several γ-pcdh variable exon promoters (γ-A2P, γ-B1P, γ-A3P, γ-C4P; see also Fig. 1A) for their responsiveness to γ-ICD, and generated luciferase reporter vectors each carrying 1 kb of the respective promoter sequences, including the conserved sequence elements (CSE) (17, 22). HEK293 cells were co-transfected with γ-A2P and either full-length EYFP-γ-pcdh-A1, EYFP-γ-pcdh-A1ΔCT, mγ-γ-ICD, or mγ-γ-ICD, and the relative light units (RLU’s) were compared in the respective cell lysates. The transactivation potentials of EYFP-γ-pcdh-A1, EYFP-γ-pcdh-A1ΔCT, and mγ-γ-ICD were not significantly different (Fig. 6A), perhaps because of insufficient processing of overexpressed EYFP-γ-pcdh-A1 in HEK293 cells. However, overexpression of mγ-γ-ICD resulted in a large increase in RLU’s (Fig. 6A).

Accordingly, for the other promoter constructs, EYFP-γ-pcdh-A1ΔCT overexpression served as the control to obtain the de-
greek of induction by γ-ICD (Fig. 6B). Results showed that all promoters were induced to similar degrees (>20-fold induction of luciferase activity) by γ-ICD, although the activity levels of the promoters differed widely (Fig. 6B). We further observed that deleting the CSE from the γ-pcdh-A2 promoter did not significantly affect the transcriptional activation by γ-ICD, although promoter activity dropped ~20-fold, in congruence with published data (22). Lastly, no significant transcriptional enhancement by γ-ICD was obtained with a luciferase reporter construct containing the core promoter sequence of the NR2C gene (28). We conclude that the γ-ICD has the potential to enhance transcription of γ-pcdh promoters, for which the CSE is not a prerequisite.

**DISCUSSION**

The γ-pcdh locus is essential for survival, as evidenced by the neonatal death of gene-targeted mice lacking it (23) or the first exon for γ-ICD (this work). Despite the importance of this locus and its widespread expression in most if not all tissues, the physiological functions of γ-pcdhs remain largely unknown. As members of the cadherin superfamily, an involvement in cell-cell interaction during development, postnatal and adult stages is predicted, but specific cell interactions dependent on subsets of γ-pcdhs have not been determined. In fact, the phenotypes observed in the mutants (19) rather suggest a role for γ-pcdhs in signaling for cell survival than in patterning.

The most striking feature of γ-pcdh locus expression, and one shared with the α-pcdh locus, is the incorporation into all γ-pcdh isoforms of an invariant cytoplasmic C-terminal domain (γ-ICD), which is likely to exert class specific signaling. The present study focused on this functional connotation. Mice were generated carrying γ-pcdh loci in which γ-C1 was deleted (ΔC1 alleles), in the expectation that this exon deletion would lead to the expression of C-terminally truncated γ-pcdh variants, rendering the γ-pcdhs similar to the β-pcdhs, which lack a constant intracellular domain (16, 17). However, the ΔC1 allele and its precursor, the neo allele, were severely expression-attenuated, as judged by RT-PCR and Northern analysis of the transcripts in hetero- and homozygous mutant mice. Most strikingly, wild-type γ-pcdh transcript and protein levels dropped to 25% in +/ΔC1 heterozygous mice instead of the 50% expected from the presence of the wild-type allele. This attenuation might reflect the scarcity of C-terminally intact γ-pcdhs in the context of an autoregulatory loop for γ-ICD-mediated locus expression. Unfortunately, no expression data on heterozygous γ-pcdh locus knockouts were reported (23), precluding comparison with +/ΔC1 mice. The attenuated expression might also reflect negative impact of products from the modified allele on the expression of the wild-type allele. The low γ-pcdh levels did however not cause systemic haploinsufficiency, as judged from the robust appearance of +/+ ΔC1 mice.

These results prompted us to study the fate and cellular location of the γ-ICD. We raised an antibody to γ-ICD and observed that subsets of neurons in sections from adult mouse brain prominently express γ-pcdhs and notably also exhibit nuclear staining. Nuclear localization of γ-ICD and the disproportionately reduced γ-pcdh expression in +/ΔC1 mice led us to investigate signaling by γ-ICD in cultured cells. Cells were transfected with various γ-pcdh constructs and subjected to pharmacological treatments to interfere with cellular pathways. The combined results made a strong case for γ-pcdhs undergoing PS-IP, with γ-secretase inhibition modifying γ-pcdh locus expression. Several classic cadherins are also subject to PS-IP (10, 11), but no nuclear translocation has been described.

The molecular mass of the putative C-terminal fragment (~20 kDa) of γ-pcdhs suggests that cleavage by presenilins occurs toward the C-terminal end of the variable exon sequences, within or shortly C-terminal of the transmembrane region. Hence, cleavage might occur within a conserved sequence stretch among the variable γ-pcdh exons. Prediction of this site is unfortunately not possible, as the presenilin cleavage sites among the known targets lack sequence conservation (42). Such lack of sequence specificity has led to the view that presenilins may initiate the degradation of transmembrane proteins, in addition to initiating specific signaling events (42). The increased γ-pcdh locus expression by recombinantly expressed γ-ICD and γ-secretase inhibition indeed suggests that γ-pcdh processing by PS-IP affects γ-pcdh locus expression. We can however not distinguish between direct and indirect transcriptional enhancement by γ-ICD, nor between repressor and activator interaction of γ-ICD. Moreover, additional changes in gene expression are likely to be produced by γ-ICD, which should be exposed by expression profiling in suitable paradigms.

Our study has revealed a vital target for γ-secretase. Death of presenilin-1/2 double knockout (dko) mice has been attrib-
Plotted as fold induction calculated as difference between transactivation potential of EYFP/H9253 and EYFP/H11022, which reflects the maximum induction (>/=20-fold). All experiments were performed in triplicate and repeated at least once. B, analysis of transactivation potential at individual 1-kb γ-VAR promoters using EYFP/H9253. The difference in relative light units between cotransfections of EYFP/H9253-ICD and EYFP/H9253-P promoter. HEK293 cells were cotransfected with vectors for the reporters and for EYFP/pcdh-A1CT. The maximum mean relative light units (mean ± S.D.) between different promoters differed widely.

To date, the clinically most interesting target of γ-secretase cleavage is APP. Aberrant γ-secretase cleavage, generated by mutations for β-secretase. Hence, unbound γ-pcdhs in the adult brain.

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REFERENCES

Fig. 6. Transactivation of γ-pcdh promoters by myc-γ-ICD. A, graphic representation of transactivation at the γ-A2P promoter. HEK293 cells were cotransfected with the luciferase reporter construct containing γ-A2P and a vector for EYFP/γ-pcdh-A1CT, EYFP/γ-pcdh-A1, or myc-γ-ICD. The difference in relative light units between cotransfections of EYFP/γ-pcdh-A1CT/γ-A2P and myc-γ-ICD/γ-A2P reflects the maximum induction (>/=20-fold). All experiments were performed in triplicate and repeated at least once. B, analysis of transactivation potential at individual 1-kb γ-VAR promoters (see Fig. 1A) and an unrelated NR2C promoter by luciferase reporter assays. HEK293 cells were cotransfected with vectors for the reporters and for EYFP/pcdh-A1CT. The maximum mean relative light units (mean ± S.D.) between different promoters differed widely.

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between cell-cell adhesive structures, could result in inaccessibility to matrix proteases and thus γ-secretase. Hence, unbound γ-pcdhs might be preferentially cleaved, thereby signaling for an increase in γ-pcdhs to strengthen cell connections.

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REFERENCES

1. Angst, B. D., Marcozzi, C., and Magee, A. L. (2001) J. Cell Sci. 114, 629–641
2. Nagar, B., Overduin, M., Ikura, M., and Rini, J. M. (1996) Nature 380, 360–364
3. Gumbiner, B. M. (2000) J. Cell Biol. 148, 399–404
4. Nagafuchi, A., and Takeichi, M. (1998) EMBO J. 7, 3679–3684
5. Togoshi, H., Abe, R., Mizeuchi, A., Takaoka, K., Chisaka, O., and Takeichi, M. (2002) Neuron 35, 77–89
6. Klemper, R. (1993) Trends Genet. 9, 317–321
7. Suzuki, S. T. (1996) J. Cell Sci. 109, 2609–2611
8. Tanaka, H., Shank, W., Phillips, G. R., Arndt, K., Bodzagi, O., Shapiro, L., Huntley, G. W., Benson, D. L., and Colman, D. R. (2000) Neuron 25, 93–107
9. Shapiro, L., and Colman, D. R. (1999) Neuron 33, 427–430
10. Marambaud, P., Wen, P. H., Dutt, A., Shioi, J., Takashima, A., Siman, R., and Robakis, N. K. (2003) Cell 114, 635–645
11. Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Ethimiospoulos, S., Zhao, Z., Wisniewski, T., and Robakis, N. K. (2002) EMBO J. 21, 1948–1956
12. Schroeter, E. H., Kissinger, J. A., and Karon, R. (1998) Nature 393, 382–386
13. Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., and Beyreuther, K. (1989) Cell 57, 115–126
14. Ni, C. Y., Murphy, M. P., Golde, T. E., and Carpenter, G. (2001) Science 294, 2179–2181
15. Brown, M. S., and Goldstein, J. L. (1997) Cell 89, 331–340
16. Wu, Q., and Maniatis, T. (1999) Cell 97, 779–790
17. Wu, Q., Zhang, T., Cheng, J. F., Kim, Y., Grimwood, J., Schmutz, J., Dickson, M., Noonan, J. F., Zhang, M. Q., Myers, R. M., and Maniatis, T. (2001) Genome Res. 11, 389–404
18. Kohmura, N., Senzaki, K., Hamada, S., Kai, N., Yasuda, R., Watanabe, M., Ishii, H., Yasuda, M., Mishina, M., and Yagi, T. (1998) Neuron 20, 1137–1151
19. Wang, X., Weiner, J. A., Levi, S., Craig, A. M., Bradley, A., and Sanes, J. R. (2002) Neuron 36, 843–854
20. Phillips, G. R., Tanaka, H., Frank, M., Klete, A., Fidler, L., Benson, D. L., and Colman, D. R. (2003) J. Neurosci. 23, 5096–5104
21. Phillips, G. R., Huang, J. K., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shank, W. S., Arndt, K., Frank, M., Gordon, R. E., Gavrinowicz, M. A., Zhao, Y., and Colman, D. R. (2001) Neuron 32, 65–77
22. Tasic, B., Nabholz, C. E., Baldwin, K. K., Kim, Y., Rueckert, E. H., Ribich, S. A., Cramer, P., Wu, Q., Axel, R., and Maniatis, T. (2002) Mol. Cell 10, 21–33
23. Wang, X., Su, H., and Bradley, A. (2002) Genes Dev. 16, 1890–1905
24. Deng, C., Thomas, K. R., and Capecchi, M. R. (1993) Mol. Cell. Biol. 13, 2134–2140
25. Nagy, A., Roseante, J., Nagy, R., Abrams-Newley, W., and Roder, J. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8424–8428
26. Schwend, P., Baron, U., and Rajewsky, K. (1995) Nucleic Acids Res. 23, 5080–5081
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27. Mack, V., Burnashev, N., Kaiser, K. M., Razov, A., Jensen, V., Hvalby, O., Seeburg, P. H., Sakmann, B., and Sprengel, R. (2001) Science 292, 2501–2504
28. Suchanek, B., Seeburg, P. H., and Sprengel, R. (1997) Biol. Chem. 378, 929–934
29. Wieden, W., and Morris, B. J. (2002) in In Situ Hybridization Protocols for the Brain, International Review of Neurobiology (Bradley, R. J., Harris, A. R., and Jenner, P., eds) Vol. 47, Academic Press, London, San Diego
30. Silver, R. A., Lue, J., Sakmann, B., and Feldmeyer, D. (2003) Science 302, 1981–1984
31. Zaborsky, L., and Heimer, L. (1989) in Neuroanatomical Tract Tracing Methods 2 (Heimer, L., and Zaborsky, L., eds) pp. 49–96, Plenum, New York
32. Zolotukhin, S., Potter, M., Hauswirth, W. W., Guy, J., and Muzyczka, N. (1996) J. Virol. 70, 4646–4654
33. Herreman, A., Hartmann, D., Annaert, W., Saftig, P., Craessaerts, K., Sernescul, L., Lums, L., Schrijvers, V., Checler, F., Vanderstichele, H., Baekelandt, V., Dressel, R., Cupers, P., Huybrechts, D., Zielin, S., Van Loven, F., and De Strooper, B. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 11872–11877
34. Herreman, A., Van Gassen, G., Bentahir, M., Nyahi, O., Craessaerts, K., Mueller, U., Annaert, W., and De Strooper, B. (2003) J. Cell Sci. 116, 1127–1136
35. Koo, E. H., and Kopan, R. (2004) Nat. Med. 10, (suppl.) S26–S33
36. Ebinu, J. O., and Yankner, B. A. (2002) Neuron 34, 499–502
37. Iwatsubo, T. (2004) Curr. Opin. Neurobiol. 14, 379–383
38. Weidemann, A., Eggert, S., Reinhard, F. B., Vogel, M., Puliga, K., Baier, G., Masters, C. L., Beyreuther, K., and Evin, G. (2002) Biochemistry 41, 2825–2835
39. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumma, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) Nature 398, 518–522
40. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selko, D. J. (1999) Nature 398, 513–517
41. Ray, W. J., Yao, M., Mumma, J., Schroeter, E. H., Saftig, P., Wolfe, M., Selkoe, D. J., Kopan, R., and Goate, A. M. (1999) J. Biol. Chem. 274, 36861–36867
42. Wolfe, M. S., and Kopan, R. (2004) Science 305, 1119–1123
43. Huppert, S. S., Le, A., Schroeter, E. H., Mumma, J. S., Saxena, M. T., Milner, L. A., and Kopan, R. (2006) Nature 445, 966–970
44. Shen, J., Bronson, R. T., Chen, D. F., Xia, W., Selkoe, D. J., and Tonegawa, S. (1997) Cell 89, 629–639
45. Peng, R., Wang, H., Wang, J., Shrom, D., Zeng, X., and Tsien, J. Z. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 8162–8167
46. Saura, C. A., Choi, S. Y., Beglopoulos, V., Malkani, S., Zhang, D., Shankaranarayana Rao, B. S., Chattarji, S., Kelleher, R. J., 3rd, Kandel, E. R., Duff, K., Kirkwood, A., and Shen, J. (2004) Neuron 42, 23–36
47. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Nature 391, 378–380
48. Murata, Y., Hamada, S., Morishita, H., Muto, T., and Yagi, T. (2004) J. Biol. Chem. 279, 3681–3682
49. Boggon, T. J., Murray, J., Chappuis-Flament, S., Weng, E., Gumbiner, B. M., and Shapiro, L. (2002) Science 296, 1308–1313