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Research Article

δ-Protocadherins: a gene family expressed differentially in the mouse brain

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Abstract. Phylogenetic analysis of protocadherin genes identified a new gene subfamily, the δ-protocadherins, containing several conserved motifs in their cytoplasmic domains. This subfamily can be further subdivided into two subgroups, named δ1-protocadherins (comprising protocadherin-1, -7, -9, and -11 or X/Y) and δ2-protocadherins (comprising protocadherin-8, -10, -17, -18, and -19). The members of the δ1-protocadherin subgroup were analyzed in greater detail here. They share a similar gene structure that results in the expression of multiple alternative transcripts. All members of this subgroup have at least one transcript that contains a binding site for protein phosphatase-1α. Like most classic cadherins, each of three δ1-protocadherins analyzed in this study by in situ hybridization showed a unique expression pattern that differed from the patterns of the other δ1-protocadherins. Together, these results suggest that the members of the δ1-protocadherin subgroup exercise tightly regulated functions in the development, regionalization, and functional differentiation of the mouse brain.

Key words. Cell adhesion; cene structure; alternative splicing; protein phosphatase-1α; brain development; expression pattern.

Cadherins constitute a large family of cell adhesion receptors that can be classified into several subfamilies, including classical cadherins, protocadherins, desmosomal cadherins, and flamingo cadherins [1]. Most cadherins studied to date are expressed in the developing nervous system, but not exclusively. In the nervous system, the expression of many cadherins is spatiotemporally regulated, and the expression of each cadherin is restricted to a specific subset of embryonic brain divisions, brain nuclei, or fiber tracts. Moreover, neurons in the same neural circuit often express the same cadherin subtype [for reviews, see refs 2–4]. In general, cadherins prefer homophilic to heterophilic interactions. Through this adhesive specificity, they contribute to the selective recognition between neuronal cells and their processes. Recent reports also indicate that particular (proto-)cadherins are expressed at synaptic contacts, and that they may be involved in regulating synaptogenesis and synaptic plasticity, both of which are important in the processes of learning and memory formation [for reviews, see refs 3, 4].

The protocadherin family has been growing dramatically in the last 10 years, and several subfamilies of protocadherins have been identified, such as the α-, β-, and γ-protocadherins, and the large (fat- and dachsous-related) protocadherins [for reviews, see refs 3, 4]. However, a large number of protocadherins do not belong to any of the protocadherin subfamilies identified to date. By a

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phylogenetic analysis of mouse and human protocadherins, to be reported in detail elsewhere, we identified a novel subfamily of protocadherins, which we named δ-protocadherins. Members of this subfamily can be divided in two subgroups, δ1 and δ2, on the basis of overall homology, number of extracellular cadherin (EC) repeats (seven versus six), and conservation of specific amino acid motifs in the cytoplasmic (CP) domains. The δ1 subgroup comprises protocadherin-1 [5], -7 [6], -9 [7], and -11(X/Y) [8]. The δ2 subgroup comprises so far protocadherin-8 [7], -10 [9], -17, -18, and -19 [10].

The aim of the present work was to analyze in more detail the gene structures, possible alternative splice forms, and expression patterns of the four members of the newly identified δ1-protocadherin subgroup in the mouse. Moreover, we have scrutinized the identity and functionality of the putative protein phosphatase-1α (PP1α) interaction domain [11] in the δ1-protocadherins.

Materials and methods

In silico identification of alternative transcripts of δ1-protocadherins

Sequences of human and mouse protocadherins-1, -7, -9, and -11 were retrieved from the public domain sequence database (NCBI) or reconstructed using Blast-N searches [12] against cDNA, expressed sequence tag (EST) and genomic libraries at EMBL and Ensembl. DNA sequence alignments were performed using the Seqman software (Lasergene; DNAstar, Madison, Wis.). Blocks, Expasy and Procite search engines were used to search for protein motifs.

Cloning of alternative CP domains of human protocadherin-11

3′ rapid amplification of cDNA ends (RACE) experiments were performed according to the Marathon cDNA amplification protocol (BD Biosciences Clontech, Palo Alto, Calif.), using human fetal brain RNA (BD Biosciences Clontech) after poly(A)+ selection (Micro-FastTrack; Invitrogen, San Diego, Calif.). 3′ RACE PCR reactions were done in final volumes of 50 µl, using the Advantage cDNA polymerase mix (BD Biosciences Clontech). Gene-specific primers (GSPs) for human PCDH11X were: (GSP1a: 5′-AAGCCCGACAGCCCTGATTTGG-3′ and GSP1b: 5′-GCACTGAAGGGGATGGCAACTC-3′) and (GSP2a: 5′-CCAAGTGTTCCTCAAGCAGTTCA-3′ and GSP2b: 5′-CTCTATGCCACA GCCCACCACT- 3′) while adaptor primers AP-1 and AP-2 were supplied by the Marathon cDNA amplification kit. RACE products were cloned in pGEMT (Promega, Madison, Wis.). Sequences were submitted to the GenBank database and are accessible under the numbers AY861432–AY861435.

Cloning of alternative CP domains of mouse δ1-protocadherins

CP domains of mouse Pcdh1, Pcdh7, Pcdh9, and Pcdh11were generated by RT-PCR cloning. For Pcdh7, BALB/c mouse brain Marathon-Ready cDNA (BD Biosciences Clontech) was used as template. For mouse Pcdh1, Pcdh9, and Pcdh11, CP domains were cloned using the Generacer system (Invitrogen). First-strand synthesis used an oligo-dT primer on total RNA from mouse brain and mouse embryo (pool of BALB/c embryos at 8.5, 11.5, 12.5, and 15.5 days post-coitum). After adaptor ligation, a first PCR reaction was done using Generacer 3′ primer and various gene-specific forward primers: GSP-Pcdh1 (5′-CAAGCAGCGTGGCAACATTCTCTT TG-3′), GSP-Pcdh9 (5′-CCGGAACATAGGAGATAG TGGTCAAC-3′) or GSP-Pcdh11(5′-GCTGGCACCAT AACTGTTGTCCTAGT-3′). Products of this first PCR were used as templates for cloning both long and short transcripts. For the second PCR, gene-specific forward primers were designed in the region encoding the juxtamembranous CP domains of the respective proteins. Their sequences (restriction sites underlined) were: GSP-F-Pcdh1 (5′-AAGGATCCAAGCCCTGGCAGTACTAG TG-3′), GSP-F-Pcdh7 (5′-AAAGAATTCACGAAGCTG GCAAAAAAGACC-3′), GSP-F-Pcdh9 (5′-AAAGATA TCAGGCCATGCGTCCAGGTTCAA-3′) and GSP-F-Pcdh11 (5′-AAAGGATCCGCAGTAAGATGCCGCCA ACCA-3′). Reverse primers for cloning the cDNA fragments encoding the short CP (sCP) domains were: GSP-R-Pcdh1-s (5′-AGAATTCGGTAAGACACACCTGCT CTATCA-3′), GSP-R-Pdh7-s (5′-AAATCTAGAGCCA AACACCGTAATGTATGG-3′), GSP-R-Pcdh9-s (5′-AA ATCTAGAACTCTACATTTGTCGGTCACC-3′) and GSP-R-Pcdh11-s (5′-AAATCTAGATATCTCACTGCA-GATTTCAATG-3′). Reverse primers for cloning the cDNA fragments encoding the longest CP (lCP) domains were: GSP-R-Pcdh1-l (5′-CGGGAATTCAGTCACAGG TAGATCTCACGCTTG-3′), GSP-R-Pcdh7-l (5′-AAAT CTAGACAGATAAACTTCTCTTCTAGTG-3′), GSP-R-Pcdh9-l (5′-AAATCTAGATTGGTGCTCCTTAGGACT CC-3′) and GSP-R-Pcdh11-l (5′-AAATCTAGAACAA GGGGTGTTCCTCTATT-3′). All PCRs were done using the proofreading polymerase Pfu (Promega). PCR fragments were sequenced and submitted to the GenBank database and are accessible under the numbers AY861416–AY861430.

Pull-down experiments with CP domains of δ1-protocadherins

Representative CP domains were cloned in appropriate Gateway entry plasmids (Invitrogen) using the restriction sites in the GSPs listed above. The cloned inserts were transferred by Gateway LR cloning into pDEST27 (Invitrogen) and pDESTcDNAmyc (constructed by rfA Gateway cassette insertion into pCS2-MT and pcDNAflag

vector fragments; vector map available upon request) in order to express fusion proteins in frame with either glutathione-S-transferase (GST) or the myc tag. This yielded for Pcdh1 the plasmids pD-GST-Pcdh1-lCP, pDcDNAmycPcdh1-lCP, pD-GST-Pcdh1-sCP, and pDcDNAmycPcdh1-sCP. Similar sets of constructs were obtained for Pcdh7, Pcdh9, and Pcdh11. For the truncated CP of Pcdh11 isoform 10, a similar myc-tagged construct was made. The CP of Pcdh9 isoform 4 (with deletion of the CM3+CM1 domain) was myc-tagged by exchange of a *Sac*I/*Bam*HI fragment in the standard Pcdh9-lCP construct. Plasmid pENTRmPP1α was constructed by ligating an *Eco*RI-*Not*I fragment from IMAGE cDNA clone 4239005 (GenBank Acc. No. BC014828), encoding isoform α of the catalytic subunit of mouse PP1, into pENTR2B vector. Gateway LR cloning yielded pD-GST-mPP1α and pDcDNAmyc-mPP1α, respectively. For pull-down experiments, HEK293T cells (1.5×10^6) were cotransfected by the calcium phosphate precipitation method with plasmids encoding, GST-fusion and myc-tagged proteins. Forty-eight hours later, cells were washed with cold PBS and lysed with cold lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, and 0.5% NP40) containing three different protease inhibitors: leupeptin, aprotinin (both from Sigma, Bornem, Belgium) and Pefablock (Pentapharm, Basel, Switzerland). The cell extracts were mixed thoroughly and centrifuged at 14,000 × g for 30 min at 4 °C. Pre-washed glutathione-Sepharose 4B beads (Amersham Biosciences, Rosendaal, The Netherlands) (50 µl) were added to the supernatant. The cell lysates were incubated with the beads for 2 h at 4 °C with end-over-end mixing. Samples were then centrifuged for 2 min at 4 °C. The supernatant was saved while the beads were washed four times with 1 ml of cold lysis buffer. Proteins were eluted with 40 µl of reduced glutathione (20 mM reduced glutathione in 50 mM Tris, pH 8.0, 0.1% Triton X-100). Eluate and supernatant (40 µl) fractions were analyzed by SDS-gel electrophoresis in 8% polyacrylamide gels followed by Western blotting. Detection was by use of both a polyclonal anti-GST goat antibody (Amersham Biosciences; dilution 1:1000) and monoclonal anti-myc tag antibody (50-fold concentrated supernatant of hybridoma clone 9E10; Developmental Studies Hybridoma Bank, Iowa City, Iowa; dilution 1:1000). Incubations and washings were as previously reported [13]. Secondary anti-goat IgG antibody coupled to horseradish peroxidase (Sigma; dilution 1:80,000) was detected by the ECL detection system (Amersham Biosciences), whereas anti-mouse IgG antibody coupled to alkaline phosphatase (Sigma; dilution 1:5000) was detected with NBT/BCIP substrate.

cRNA probe preparation

The mouse Pcdh7 probe plasmid comprises a 1.6-kb PCR fragment encoding part of the EC domain, starting at the beginning of EC repeat 3 (forward primer: 5′-GCTGACCTGGCTGAGAACA-3′) and ending right after EC repeat 6 (reverse primer: 5′-CCTGGGTGAGTGGGGTATG-3′). The mouse Pcdh9 probe plasmid also comprises a PCR fragment encoding part of the EC domain, starting in the middle of EC-3 (forward primer: 5′-GAAGAGACAGCCATTCAC-3′) and ending in the middle of EC-7 (reverse primer: 5′-CCAGGTCACTTATGTTGA-3′). The probe plasmid for mouse Pcdh11 comprises a PCR fragment encoding part of the EC domain, starting in the middle of EC-4 (forward primer: 5′-CCTTTCAGATTGCGACCAGTAT-3′) and ending after the transmembrane (TM) domain (reverse primer: 5′-TGGTTGGCGGCATCTTACTA-3′) (see also 'cRNA probe' in fig. 2). These clonings were done using the proofreading polymerase Pfu (Promega) with mouse brain Marathon-Ready cDNA (Promega) as template, followed by ligation into the pGEM-Teasy vector (Promega). Probe specificity was confirmed by cDNA colony hybridization (data not shown).

The following plasmids were also used: mcad8-12 containing a 1.6-kb fragment of mouse cadherin-8 cDNA from the 5′ region [14], and pBSMR4 containing full-length mouse cadherin-4 (R-cadherin) cDNA [15], a kind gift from Drs. H. Matsunami and M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan).

RNA synthesis using T7 or SP6 RNA polymerase (Riboprobe Labelling Kit; Promega) was performed according to the manufacturer's protocols, using digoxygenin-labeled ribonucleotides. Labeled probes were precipitated with 3 M NaAc (pH 6.0) and ethanol and resuspended in TE buffer. Testing of incorporation was done by RNA gel electrophoresis followed by Northern blotting and Western detection with anti-digoxygenin antibody.

In situ hybridization

A previously published protocol was followed [16]. According to national and institutional guidelines for the care of animals in research, post-natal mice were killed by decapitation. Immediately following dissection, tissue was fixed in 4% formaldehyde (FA) in phosphate-buffered saline (PBS) and dehydrated in solutions of increasing concentrations of sucrose in PBS. Tissues were embedded in TissueTek O.C.T. compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Twenty-micrometer-thick sections were obtained using a refrigerated microtome (Microm International, Walldorf, Germany). Sections were dried, post-fixed in 4% FA/PBS and treated with proteinase K. After post-fixation, sections were incubated in a 0.25% acetic-anhydrate solution to saturate non-specific RNA binding. Sections were hybridized overnight with sense or antisense cRNA probe at 56 °C. Following hybridization, sections were washed in 50% formamide in 2× SSC and treated with RNase A. Sections were then washed again in 50% for-

mamide in 2× SSC (60 °C for 30 min), in 2× SSC (55 °C for 30 min), in 0.1× SSC (30 min at room temperature) and twice in PBS. The digoxygenin-labeled probe was detected with sheep anti-digoxygenin antibody Fab fragments conjugated with alkaline phosphatase (Boehringer, Mannheim, Germany). For sense probes, no significant staining was observed (data not shown). Anti-sense probes for all (proto-)cadherins investigated resulted in strong labeling of some unique regions for each probe, indicating a high degree of probe specificity. For neuroanatomical orientation, adjacent brain sections were stained with 0.1% thionine solution. Sections were viewed under a transmission light microscope (BX40; Olympus, Hamburg, Germany) equipped with a computer-based digital camera (DP70; Olympus). Photomicrographs were taken on transmission light microscopes (Ultraphot or Axiophot; Zeiss, Oberkochen, Germany) equipped with conventional cameras. Images were digitized, enhanced in contrast, and adjusted in brightness, if required, and labeled with the Photoshop software (Adobe Systems, Mountain View, Calif.).

Results

Two novel subgroups of protocadherins identified by phylogenetic analysis

In total, cytoplasmic (CP) domains of 70 different protocadherins were aligned in a phylogenetic analysis. These comprised the majority of the known human and mouse unclustered protocadherin genes, plus four members only of each of the clustered α-, β-,and γ-protocadherin gene families to reduce complexity. The resulting phylogenetic analysis will be reported elsewhere, but confirmed the existence of the following previously identified protocadherin subfamilies: α-, -β-, and γ-protocadherin subgroups; large (Fat- and Dachsous-like) cadherins; and the seven-pass transmembrane (CLR or flamingo-like) cadherins [for reviews, see refs 1, 3, 4, 17, 18]. In addition, one group of nine human protocadherins (PCDH-1, -7, -8, -9, -10, -11, -17, -18, and -19) and their mouse orthologues could be clearly distinguished from the remaining protocadherins, although their mutual homology is not that high. Wolverton and Lalande [10] showed that all these proteins belong to a subfamily of protocadherins that contain two conserved motifs in their CP domains, called CM1 and CM2. The CM2 motif is highly conserved in the nine members, while the CM1 motif is less conserved but still clearly distinguishable, as is evident from figure 1. We propose the term δ-protocadherins for this subfamily of protocadherins.

A more detailed analysis strongly suggests that the δ-protocadherin family can be divided into two distinct groups. One group, here called the δ1-protocadherin subgroup, comprises the protocadherins-1, -7, -9, and -11 in both human and mouse. Remarkably, these are also the only known protocadherins containing seven EC domains [4]. Figure 1 shows that each of these four δ1-protocadherins contains a short but conserved motif (RRVTF) in their CP domain, which we call the CM3 motif. This motif has previously been shown to be necessary for the binding between human PCDH7 and PP1α [11], a cytosolic phosphatase believed to be important in synaptic plasticity [for a review, see ref. 19]. The remaining members of the δ-protocadherin subfamily (protocadherins-8, -10, -17, -18, and -19), here called the δ2-protocadherin subgroup, are also clustered together in the phylogenetic tree. These protocadherins contain six EC repeats and do not have a CM3 motif in their CP domain (fig. 1).

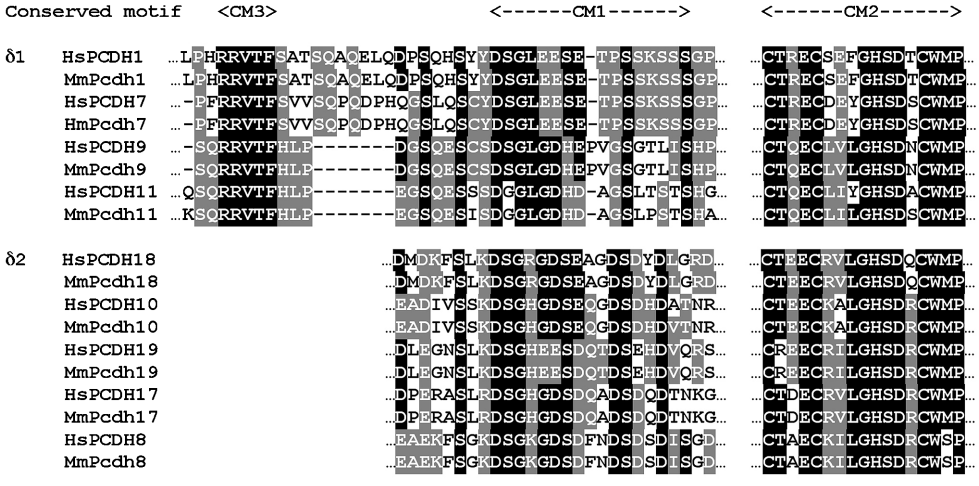


Figure 1. Multiple amino acid alignment of selected parts of the cytoplasmic domains of the nine δ-protocadherins in human and mouse, showing the conserved motifs. Note that the CM1 domain is less conserved than the CM2 domain. The CM3 motif is perfectly conserved within the δ1-protocadherins but absent in the δ2 subfamily. Upstream and downstream sequences are not shown to reduce the complexity of the figure. Hs, *Homo sapiens*; Mm, *Mus musculus*.

Moreover, the CM1 motif in this group is more highly conserved than the corresponding motif in the δ1-protocadherin subgroup.

Similarities in structure of the δ1-protocadherin genes

To ascertain the validity of the above classification, we unraveled the gene structure of the four members of the δ1-protocadherin subgroup in both human and mouse. Despite the different chromosomal locations of all four genes, they have similar, though not identical, structures, with extensively shared exon-intron boundaries and exon/intron lengths (fig. 2, and tables S1 and S2 of Supplemental Material, available on URL: http://www.dmbr.ugent.be/root/contents/publications/supplementary.html).

The human PCDH7 and PCDH9 genes are localized on chromosome 5p15 [6] and chromosome 13q14-21 [7], respectively. In both human and mouse, the standard open reading frames (indicated by * in fig. 2) have a rather simple structure, comprising a single large 5′ exon encoding the complete EC and TM domains and part of the CP domain, followed by a small exon of approximately 200 bp containing the conserved CM3 and CM1 motifs, and a larger 3′ exon encoding the 3′ tail of the CP domain, comprising CM2 (fig. 2B, C; table S2).

The human PCDH1 gene is localized on chromosome 5q32-33 [20], close to the region of the clustered α-, β-, and γ-protocadherin genes. Its standard structure is more complex and contains five coding exons, of which the first three encode the EC, TM, and part of the CP domains. These three exons are then followed by a smaller exon of approximately 200 bp, again encoding CM3 and CM1, and a larger 3′ exon comprising CM2 (fig. 2A; table S2). The reported start codon of the human PCDH1 gene (depicted as ATG1 in fig. 2A) is not conserved in the protocadherin-1 genes of chimpanzee, mouse, rat, and dog, whereas the first in-frame ATG in the mouse Pcdh1 gene shows a better Kozak consensus sequence [21], and is conserved in all species mentioned including human (ATG2 in fig. 2A). This suggests that the latter ATG is used preferentially in the human PCDH1 gene as well.

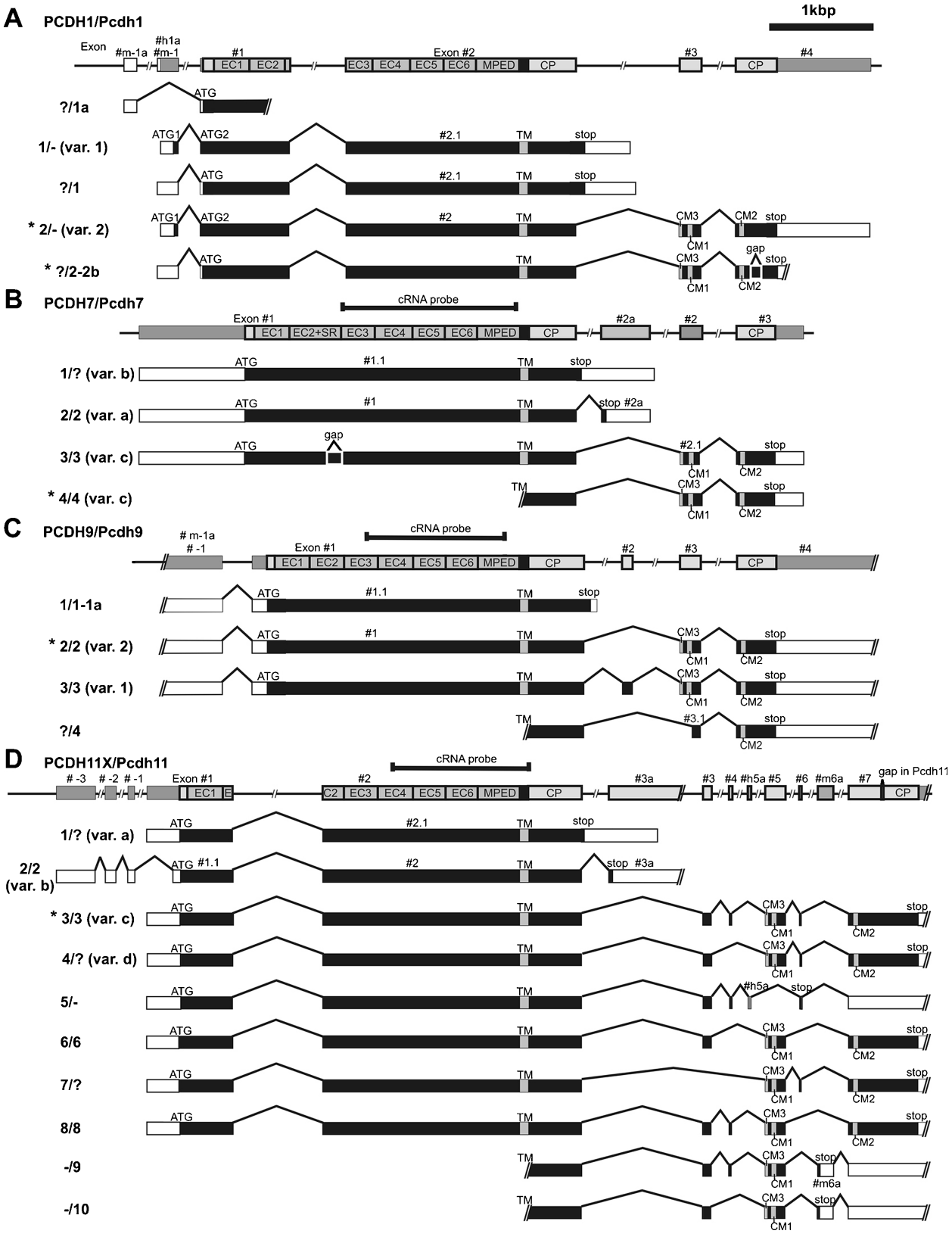
The human PCDH11X gene is localized in the hominid-specific XY-homology region on chromosome Xq21.3 [22], and the highly related human PCDH11Y gene on chromosome Yp11.2 [8]. PCDH11X genes in human and mouse have far more complicated structures, as shown previously [23, 24]. The PCDH11Y gene shows similar complexity but will not be dealt with in this report as there is no homologue for it on the mouse Y chromosome. Our results largely confirm the standard PCDH11X gene structure containing seven coding exons, preceded by several non-coding exons (fig. 2D, tables S1, S2). Contrary to Blanco-Arias and coworkers [24], we have adopted the convention of starting exon numbering with the first coding exon. This allows a more straightforward comparison with the other δ1-protocadherin family members. The first two coding exons (exon #1 and #2) of PCDH11/Pcdh11encode the EC, TM, and part of the CP domain. The remainder of the CP domain is encoded by a diverse subset of coding exons, and the longest CP domain variant is encoded by five more coding exons. As in the other three δ1-protocadherin genes, a small exon of approximately 200 bp (exon #5) encodes the CM3 and CM1 motifs. Additional exons are even smaller (ranging from 24 to 81 bp). The last 3′ exon (exon #7) encodes a large part of the PCDH11 CP tail and contains the CM2 motif.

When comparing the four gene structures, the following similarities become evident. First, in all four genes, the complete EC domain, TM domain, and a short part of the CP are encoded by not more than one to two large 5′ exons. The protein fragment encoded by these 5′ exons ends at a position conserved in the four δ1-protocadherin types. Second, following these 5δ1 exons, the four genes have a conserved small exon of approximately 200 bp. The protein sequence encoded by this exon starts immediately with the CM3 and CM1 motifs. Third, the remaining parts of the CP domains (containing the CM2 motif) of PCDH1, PCDH7, and PCDH9 are encoded by single exons (fig. 2A–C) that are highly similar at the protein level. The 3′ part of the PCDH11X gene shows similarity to the 3′ parts of the other three genes of the subgroup, but it comprises many more exons (fig. 2D). Nevertheless, exons #5 and #7, encoding the various CM motifs, are quite similar to those of the other δ1-protocadherin family members.

Evidence for alternative splicing in all eight genes

Alternative transcripts have been reported for human and mouse PCDH7/Pcdh7 [6], for human PCDH9 [7], and particularly for human PCDH11X and PCDH11Y [8, 22, 24]. Based on the gene structures described above, we identified new transcripts for all δ1-protocadherin genes in mouse and human (fig. 2, table S2). Evidence for the occurrence of many of these alternative transcripts was obtained by screening the coding sequence against the human and mouse EST libraries, and by RT-PCR and 3′ RACE experiments with GSPs (table S2; GenBank Acc. No. AY861416–AY861435).

Several alternative transcripts of the human and mouse δ1-protocadherin genes show remarkable similarity (fig. 2; table S2). The shortest transcripts of all genes encode a truncated transmembrane protein lacking the conserved motifs CM1–CM3 in their CP domain. Several of these transcripts (isoform 1 in fig. 2) result from intronic readthrough, following the first (for human PCDH7 and -9, and for mouse Pdh9), first two (for human PCDH11X and for mouse Pcdh1) or first three (human PCDH1) coding exons. In the transcript of isoform


A
PCDH1/Pcdh1
1kbp
Exon
#m-1a
#h1a
#m-1
#1
EC1
EC2
Exon #2
EC3
EC4
EC5
EC6
MPED
CP
#3
#4
?/1a
ATG
1/- (var. 1)
ATG1
ATG2
#2.1
TM
stop
?/1
* 2/- (var. 2)
#2
CM3
CM2
CM1
* ?/2-2b
gap
B
PCDH7/Pcdh7
cRNA probe
Exon #1
EC2+SR
#2a
#2
#3
1/? (var. b)
#1.1
2/2 (var. a)
stop #2a
3/3 (var. c)
4/4 (var. c)
C
PCDH9/Pcdh9
m-1a
-1
1/1-1a
* 2/2 (var. 2)
3/3 (var. 1)
?/4
#3.1
D
PCDH11X/Pcdh11
-3
-2
-1
E
C2
#3a
#4
#h5a
#5
#6
#m6a
#7
gap in Pcdh11
1/? (var. a)
2/2 (var. b)
#3a
* 3/3 (var. c)
4/? (var. d)
5/-
6/6
7/?
8/8
-/9
-/10

2 of PCDH7 and Pcdh7, no readthrough is observed but there is splicing to an alternative splice acceptor site in the intron following the exon that encodes the TM domain, resulting in alternative exon #2a bearing a premature stop codon. The same holds true for use of exon #3a in type 2 isoforms of PCDH11X and Pcdh11. In any case, the position of this first exon-intron boundary 3′ of the TM-encoding sequence is remarkably conserved. The larger transcripts, indicated by asterisks in figure 2 and coding for all three CMs in the respective CP domains, are the products of what we believe to be standard splicing to the additional 3′ exons. However, readthrough of the CM3-encoding exon #2 is observed in mouse Pcdh7 (resulting in alternative exon #2.1 for isoform 4; fig. 2B). For exon #3 of mouse Pcdh9, an alternative intraexonic splice acceptor is observed (resulting in alternative exon #3.1 for isoform 4; fig. 2C). Also, between the TM-encoding exon and the CM3-encoding exons, alternatively used exons are found for PCDH9/Pcdh9 (exon #2) and for PCDH11X (exon #h5a). The use of exon #h5a, which has not been reported before but is present in both chromosomes X and Y of the human genome although not in the mouse genome, results in a premature stop codon and generation of transcript isoform 5, not containing any of the CM motifs (fig. 2D). Exons #4 and #6 are skipped in some protocadherin-11 transcripts, both in human and mouse (e.g. isoforms 4, 6, or 8). In mouse Pcdh11 transcripts, use of alternative exon #m6a, which is not conserved in the human genome, results in a premature stop codon and loss of the CM2 motif (isoforms 9 and 10 in fig. 2D). In conclusion, although we have confirmed by 3′ RACE experiments the occurrence of several alternative PCDH11 transcripts that have been reported in the public domain [see also ref. 24], we have also identified several additional alternative transcripts in both human and mouse protocadherin-11 transcripts (fig. 2D, table S2). In total, ten different transcripts were found in either mouse or human for PCDH11/Pcdh11.

A common interaction partner of δ1-protocadherins

The CM3 motif that comprises the RRVTF motif reported to be necessary for the interaction between human protocadherin-7 (variant c or isoform 3; fig. 2C) and PP1α [11] turned out to be specific for δ1-protocadherins (fig. 1) as it is not found in any of the δ2- or non-δ-protocadherins.

We therefore addressed the question whether the other δ1-protocadherins could also interact with PP1α through the conserved CM3 domain. For use in pull-down experiments, we cloned both short and standard long (asterisks in fig. 2) CP domains of the four mouse δ1-protocadherins in frame with either an amino-terminal GST protein or an amino-terminal myc tag in eukaryotic expression vectors. The occurrence of natural splice variants lacking particular CM domains (Pcdh9 isoform 4 and Pcdh11 isoform 10; fig. 2C, D) was exploited to determine specificity of interactions. HEK293 cells were cotransfected with these constructs and a plasmid encoding myc-tagged mouse PP1α. Affinity-purified GST-fusion proteins were analyzed by SDS-PAGE followed by Western blotting for the myc tag (fig. 3). We could confirm the interaction of PP1α with the standard long CP of Pcdh7 [11], whereas the short CP of Pcdh7 showed no significant interaction (data not shown). Similar findings were obtained for the short and long CP domains of Pcdh1, Pcdh9, and Pcdh11. Representative data are shown in figure 3 A–C. Some

Figure 2. Gene structure and alternative transcripts of the four members of the protocadherin-δ1 subgroup. Results are basically identical for human (isoform numbers before slash signs) and mouse (isoform numbers after slash signs) although significant differences exist. If a particular transcript does not exist in one of the two species, this is indicated by a minus sign before or after the slash sign. Variant annotations between parentheses refer to the literature on mainly human protocadherins, but lack overall consistency. Question marks denote presumable transcripts that need to be validated experimentally (cloned) in one of the two species. Asterisks refer to 'standard' long transcripts. Exons are drawn to scale and numbered as indicated. Exon #1 is defined to be the exon that contains the standard start codon (indicated by ATG); alternatively used exons are indicated by the extension 'a'; exons that are elongated by readthrough in the neighboring intron or that become shortened by an intraexonic splice acceptor are indicated by the extension '.1.' Exons that occur only either in the human or mouse genome are indicated by h and m, respectively. Gray boxes represent exons, the intervening line represents intronic sequence (the latter not at scale). Black boxes represent coding sequences in the transcripts, white boxes represent 5′ or 3′ untranslated regions. EC, sequences encoding the extracellular cadherin repeats; TM, sequence encoding the transmembrane domain; CM3, CM1, and CM2, conserved motifs; SR, serine-rich region. Hybridization probes used in this study are depicted above the respective genes. (*A*) Protocadherin-1 genes (human PCDH1, mouse Pcdh1) and derived transcripts: 1, truncated transcript; 2, standard transcript. According to the literature [5], the human protein starts with the ATG in exon #1a, which is not conserved in mouse or other species. In contrast, the first in-frame ATG in exon #1 is conserved across many species including human and shows a better Kozak consensus sequence. (**B**) Protocadherin-7 genes (human PCDH7, mouse Pcdh7) and derived transcripts: 1 and 2, two truncated transcripts, only differing at the 3′ end; 3, differs from the standard transcript (4) by truncated exon 2. (*C*) Protocadherin-9 genes (human PCDH9, mouse Pcdh9) and derived transcripts: 1, short transcript; 2, standard transcript; 3, transcript with additional exon #2; 4, transcript with variant exon #3.1, lacking CM3 and CM1. (*D*) Protocadherin-11 genes (human PCDH11X, mouse Pcdh11) and derived transcripts: 1 or a, short CP domain; 2 or b, use of alternative exon #3a; 3 or c, full-length transcript; 4 or d, idem without exon #4; 5, use of alternative human exon #h5a resulting in frameshift; 6, neither exon #4 nor exon #6; 7, neither exon #3 nor exon #4; 8, no exon #6; 9, use of alternative mouse exon #m6a, resulting in premature stop codon and loss of CM2; 10, as transcript 9 but with exon #4 skipped. For details of the exon/intron boundaries and for evidence of transcription, see Supplementary Material available on URL: http://www.dmbr.ugent.be/root/contents/publications/supplementary.html.

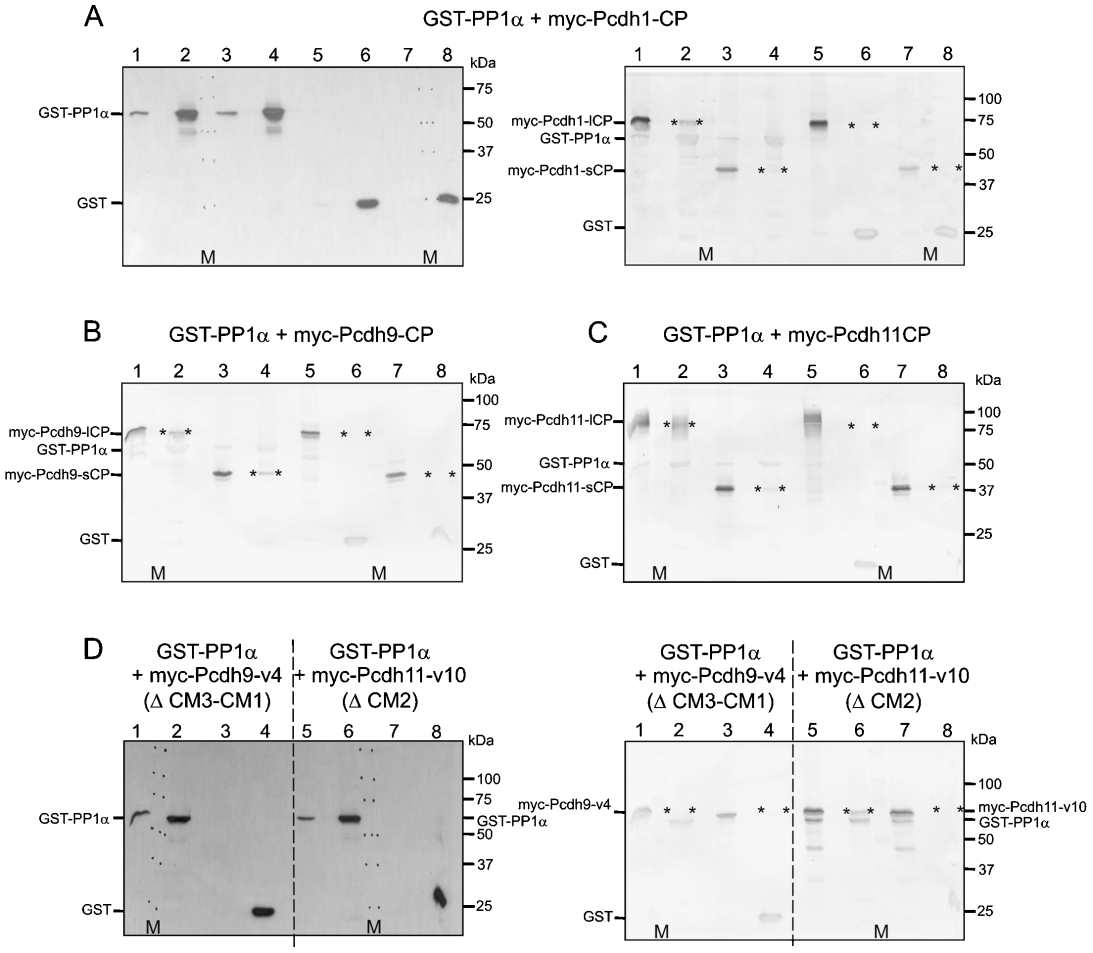


Figure 3. Interaction of PP1α with long forms of cytoplasmic domains (CPs) of δ1-protocadherins through the conserved CM3 domain. Pull-down experiments were performed as described in Materials and methods. (*A–C*) HEK293 cells were cotransfected with plasmids, encoding a GST-fusion protein of mouse PP1α (lanes 1–4) or GST alone (lanes 5–8) in combination with constructs encoding either standard long (lCP; lanes 1, 2, 5, 6) or short (sCP; lanes 3, 4, 7, 8) forms of CPs of mouse δ1-protocadherins all in frame with an amino-terminal myc tag. Analysis was performed for CPs of Pcdh1 (*A*), Pcdh9 (*B*), and Pcdh11 (C). (*D*) HEK293 cells were cotransfected with plasmids, encoding a GST-fusion protein of mouse PP1α (lanes 1 and 2) or GST alone (lanes 3 and 4) in combination with constructs encoding the CP of either isoform 4 of Pcdh9 (lanes 1–4) or isoform 10 of PCdh11 (lanes 5–8), both in frame with an amino-terminal myc tag. Affinity-purified GST-fusion proteins were analyzed by SDS-PAGE followed by Western blotting for GST (left panels in *A* and *D*; anti-GST antibodies revealed by ECL; not shown for Pcdh9 and Pcdh11) and the myc tag (right panels in *A* and *D*, panels *B* and *C*; anti-myc antibodies revealed by phosphatase reactions, with slight detection of anti-GST antibodies as well). Asterisks flank the position of pulled down protocadherin fragments. M, lanes marked with molecular-weight markers as indicated on the right. Long CPs consistently associated with PP1α but not with GST alone, whereas short or variant CPs lacking at least the CM3+CM1 domain showed very weak or no association.

interaction was also seen between the short CP and PP1α (lanes 4), but this was always much less explicit than for the interaction with long CP (lanes 2). Moreover, the CP of Pcdh9 isoform 4, a natural variant that lacks the CM3+CM1 motifs, failed to bind PP1α (fig. 3D, lanes 1–4), in contrast to the CP of Pcdh11 isoform 10, a natural variant that lacks the CM2 motif (fig. 3D, lanes 5–8). This unequivocally identified the CM3+CM1 domain as the major PP1α interaction site for all the δ1-protocadherins.

Analysis of differential expression patterns of protocadherin-7, -9, and -11 in the nervous system

All four δ1-protocadherin genes were reported to be expressed at high levels in the nervous system [5–8, 11, 22]. We therefore investigated their expression patterns in more detail by an in situ hybridization study.

Specific cRNA probes for Pcdh7, Pcdh9, and Pcdh11 covered sequences encoding part of the EC domain and ending either just in front of the TM domain (for Pcdh7 and Pcdh9), or right behind the TM domain (for Pcdh11) (see also 'cRNA probe' in fig. 2). As a result, both short and long isoforms were detected. An appropriate Pcdh1 probe was not available to us at the time of these experi-

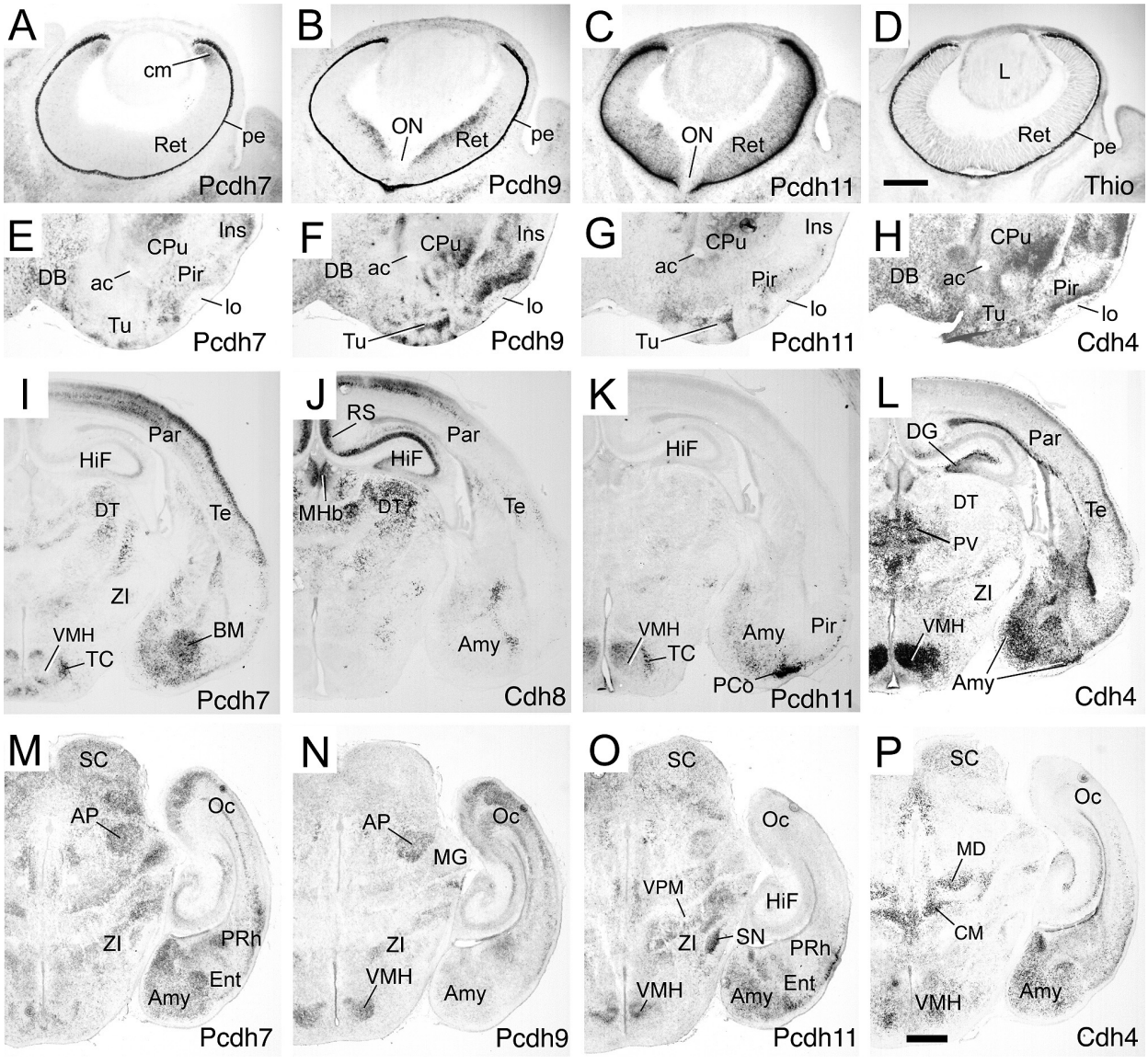


Figure 4. (*A–D*) Transverse sections through the E15 mouse eye, hybridized with anti-sense probes for protocadherin-7 (Pcdh7; *A*), protocadherin-9 (Pcdh-9; *B*), and protocadherin-11 (Pcdh11; *C*), and thionine (Thio) stain of an adjacent section (*D*). Note the differential expression of the three protocadherins in the neural retina (Ret). The dark staining in the pigment epithelium (pe) represents pigment rather than hybridization signal. (*E–P*) Coronal sections through the P1 mouse forebrain at the level of the olfactory tubercle (Tu; *E–H*), the temporoparietal cortex (Te, Par; *I–L*), and the occipital cortex (Oc; *M–P*) hybridized with anti-sense probes for protocadherin-7 (Pcdh7; *E*, *I*, *M*), protocadherin-9 (Pcdh9; *F*, *N*), protocadherin-11 (Pcdh11; *G*, *K*, *O*), cadherin-8 (Cdh8; *J*), and cadherin-4 (Cdh4; *H*, *L*, *P*). Note the differential expression patterns of the three protocadherins in forebrain nuclei and in telencephalic cortical regions and layers, which is comparable, in general, to the differential expression of the classic cadherins (Cdh4 and Cdh8). Other abbreviations: ac, anterior commissure; Amy, amygdaloid complex; AP, pretectal area; BM, basomedial amygdaloid nucleus; cm, ciliary margin; CM, centromedial thalamic nucleus; CPu, caudate-putamen (striatum); DB, diagonal band; DG, dentate gyrus; DT, dorsal thalamus; Ent, entorhinal cortex; HiF, hippocampal formation; Ins, insular cortex; L, lens; lo, lateral olfactory tract; MD, mediodorsal thalamic nucleus; MG, medial geniculate nucleus; MHb, medial habenula; ON, optic nerve exit point; PCo, posterior cortical amygdaloid nucleus; Pir, piriform cortex; PRh, perirhinal cortex; PV, paraventricular thamalic nucleus; RS, retrosplenial nucleus; SC, superior colliculus; SN, substantia nigra; TC, tuber cinerum area; VMH, ventromedial hypothalamic nucleus; VPM, ventral posteriomedial thalamic nucleus; ZI, zona incerta. Scale bars, 0.2 mm (in *D* for *A–D*), and 0.5 mm (in *P* for *E–P*).

ments. In situ hybridization with digoxigenin-labeled sense and anti-sense RNA probes was performed on serial sagittal sections of whole mouse embryos and dissected mouse embryonic brain after 15 and 17 days of gestation (E15, E17). Transverse sections of E15 retinae and postnatal day 1 (P1) brains were also hybridized. For comparison, adjacent sections were hybridized with probes for cadherin-4 (Cdh4, R-cadherin) and cadherin-8 (Cdh8).

All three protocadherins were prominently expressed in the nervous system (figs. 4, 5). However, a number of

other organs also exhibited differential expression of the protocadherins (to be reported in detail elsewhere). As in the case of most classic cadherins, the expression of all three protocadherins was restricted to specific brain nuclei, and cortical regions and layers in the brain. Each protocadherin had a unique expression pattern that differed from the patterns of other protocadherins, and also from those of Cdh4 [25] and Cdh8 [14]. For example, in the E15 retina (Ret), Pcdh7 was expressed in the proliferative ciliary margin (cm), Pcdh9 was expressed in the inner, prospective ganglion cell layer, and Pcdh11 was expressed in all retinal layers (fig. 4A–D). The vomeronasal organ, the olfactory bulb, and the accessory olfactory bulb expressed Pcdh7 and Pcdh9 differentially (data not shown). In the P1 telencephalon, the three protocadherins were expressed in different layers and regions of the cerebral cortex (RS, Par, Te, Oc, Ins, Ent, Pir) and hippocampus (Hi; figs 4E–P, 5). In the caudate-putamen (CPu), the three protocadherins were expressed by the matrix of different striatal regions (fig. 4E–G). The dorsal pallidum contained neurons that expressed Pcdh11 strongly (GP in fig. 5A) and Pcdh7 moderately (data not shown). In ventral pallidal regions, e.g., the amygdaloid complex (Amy) and the olfactory tubercle (Tu), there were patchy expression patterns that differed among all three protocadherins and were related to histological differentiation in these regions (figs. 4E–P, 5A–C). The brain nuclei of the diencephalon (figs. 4I–P, 5A–D), mesencephalon (figs. 4M–P, 5D) and hindbrain (data not shown) had a complex, differential expression of all three protocadherins.

In conclusion, the expression patterns of the three protocadherins were distinct and regionally restricted to specific anatomical and functional structures of the developing brain.

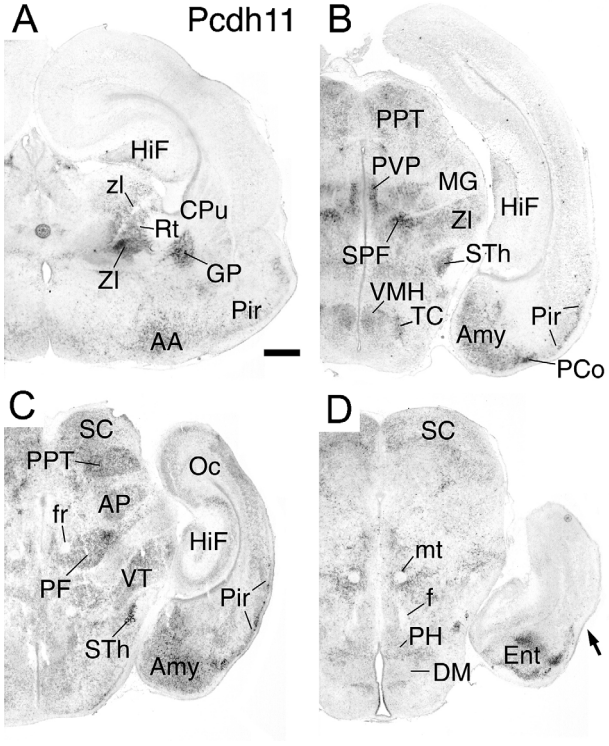


Figure 5. Coronal sections of P1 mouse forebrain hybridized with anti-sense probe for protocadherin-11 (Pcdh11). Sections are arranged in a rostral to caudal sequence and were taken at the level of the globus pallidus (GP; *A*, at a level in between the sections displayed in fig. 4G, K), of the subthalamic nucleus (STh; *B*, at a level in between the sections displayed in fig. 4K, O), of the occipital cortex (Oc; *C*, at a level in between the sections displayed in panel B and fig. 4O), and of the entorhinal cortex (Ent; *D*, at a level caudal to the section displayed in fig. 4O). The arrow indicates the rhinal fissure. Other abbreviations: AA, anterior amygdaloid area; Amy, amygdaloid complex; AP, anterior pretectal area; CPu, caudate-putamen (striatum); DM, dorsomedial hypothalamic nucleus; f, fornix; fr, retroflex fascicle; HiF, hippocampal formation; MG, medial geniculate nucleus; mt, mamillotegmental tract; PCo, posterior cortical amygdaloid nucleus; PF, parafascicular thalamic nucleus; PH, posterior hypothalamic area; Pir, piriform cortex; PPT, posterior pretectal nucleus; PVP, paraventricular thalamic nucleus, posterior part; Rt, reticular thalamic nucleus; SC, superior colliculus; SPF, subparafascicular thalamic nucleus; TC, tuber cinerum area; VMH, ventromedial hypothalamic nucleus; VT, ventral thalamus; ZI, zona incerta; zl, zona limitans. Scale bar, 0.5 mm.

Discussion

The δ-protocadherin family of molecules comprises two distinct subgroups

To enable classification, we performed a phylogenetic analysis of the cytoplasmic domains of most known protocadherin genes in mouse and human. Our results (to be reported in detail elsewhere) confirmed the identification of several previously identified subfamilies [1, 3, 4]. In addition, our analysis revealed the existence of two novel subgroups within the subclass of protocadherins previously described by Wolverton and Lalande [10], and designated here as δ-protocadherins. All members of this subfamily contain the motifs CM1 and CM2 in their cytoplasmic domains [10]. The first subgroup within the δ-protocadherins comprises protocadherins-1, -7, -9, and -11(-X/Y); these protocadherins were designated δ1-protocadherins and contain, in addition to the CM1 and CM2 motifs, an additional conserved cytoplasmic motif, here termed the CM3 motif. The latter is adjacent to the CM1 motif. The membrane-proximal cytoplasmic domain preceding the CM motifs is also highly conserved throughout the δ1-protocadherin family members. Moreover, the δ1-protocadherins are the only known protocadherins with seven EC repeats. The second subgroup, called δ2-protocadherins, comprises protocadherins-8, -10, -17, -18, and -19. This subgroup lacks the CM3 motif; in contrast, the CM1 and CM2 motifs exhibit relatively high conservation within this subgroup, and all members have six EC repeats. Our subsequent work

described in this report focused on the members of the δ1-protocadherin subgroup.

δ1-Protocadherins have a conserved but not identical gene structure, resulting in multiple transcripts

Although the gene structures of the four members of this family are not identical, there are several interesting similarities. First, the exon encoding in each case (part of) the EC domain, the TM, and a 5′ part of the C stops at about the same position. This appears to be a common feature for protocadherins, as most protocadherin genes reported so far have an exon-intron boundary in the 5′ part of the sequence encoding the cytoplasmic tail. The remaining part of the cytoplasmic tail can be encoded by one to several exons, differing in length and number throughout the protocadherin family [3, 17].

Second, the four genes all contain an exon of about the same length, encoding the part of the cytoplasmic domain that comprises the CM3 and CM1 motifs. The last part of the cytoplasmic domain is encoded by a larger exon, and contains the CM2 motif.

Remarkably each δ1-protocadherin gene yields multiple alternative transcripts. Only the longer transcripts contain the CM3, CM1, and CM2 motifs. In rare cases, only CM2 or the CM3-CM1 doublet are represented (fig. 2; transcript Pcdh7-4, transcripts Pcdh11-9 and -10). The synthesis of the shorter isoform 1 transcripts resembles that of the carboxy-terminal ends of many β-protocadherin family members [26] and the O-type transcripts of the α-protocadherin family members [27]. We propose that these short transcripts are the result of intronic readthrough, leading to the formation of truncated proteins that might be unable to interact with still-to-be-identified cytoplasmic proteins. The isoform 2 transcripts of PCDH7/Pcdh7 and PCDH11/Pcdh11 (fig. 2) might be variants on the same theme; they encode truncated proteins formed by splicing to a 3′ exon encoding a short C-terminal peptide lacking the CMs. All these truncated proteins might function in a dominant-negative way with respect to CM-mediated functions still to be elucidated.

The human PCDH11X and mouse Pcdh11 genes are most remarkable with regard to expression of multiple alternative transcripts [8, 24]. The complete gene spans a region of about 790 kb in human and over 610 kb in mouse. The transcripts encoding the long standard version of the cytoplasmic domains are formed by the splicing of seven exons, of which three are very small (81, 30, and 24 bp). Several alternative transcripts are the result of in-frame exon skipping, and the encoded proteins do not differ that much from the standard full-length protein, as they still comprise the three CMs. Some transcripts, however, are generated from alternatively used exons (#3a in both human and mouse, #h5a in human, and #m6a in mouse), causing frameshifts and resulting in truncated proteins. The number of alternative transcripts for the human and mouse Pcdh11 genes reported in the present work significantly extends the reported data on splice variants of the human PCHD11X transcripts [8, 24]. Moreover, this is the first time that splice variants have been reported for the mouse Pcdh11 gene.

The human PCDH11X gene is localized in the hominid-specific XY-homology region. Translocation of this region from the X chromosome to the Y chromosome is believed to have been the starting point of human speciation about 100,000–150,000 years ago. The presence of a transcribed protocadherin gene, believed to be important in brain development, on both the X and Y chromosomes [8] suggests that this gene is a candidate key player in human speciation, particularly in terms of cerebral asymmetry and language formation, two human-specific characteristics [28]. A role in sexual selection has also been discussed [8]. The multiple small exons of PCDH11X and PCDH11Y, and their separation by huge introns, makes these genes vulnerable to mutations and erroneous transcript formation, possibly causing neural diseases [8, 28]. However, one recent study did not find sequence variations in this gene in schizophrenia or schizoaffective disorders [29].

Common interaction partners for δ-protocadherins?

Our results demonstrate that the CP domains of all four δ1-protocadherin proteins contain the RRVTF sequence (CM3) that was initially shown for human protocadherin-7 to interact with PP1α [11]. Apart from the interaction of the protein kinase Fyn with the CNR protocadherins [30], no other common cytoplasmic interaction partners for protocadherin subfamilies have been identified to date. PP1α is a brain-enriched protein that is expressed specifically in dendritic spines of several neuron types in the brain [for a review, see ref.19]. PP1α has been implicated in regulating synaptic plasticity, including long-term potentiation and long-term depression [31–33], both key processes in memory formation and learning. Yoshida and coworkers [11] demonstrated that the PP1α enzymatic function is inhibited upon binding to the cytoplasmic tail of protocadherin-7. In view of the previously described functions of PP1α in the nervous system, the interaction with δ1-protocadherin molecules, differentially expressed in the nervous system, could provide a new link between cell-cell adhesion or recognition and intracellular signaling in neuronal cells. Other possible interaction partners for members of the δ-protocadherin subfamily include TAF-1/set, a protein involved in cell cycle regulation. TAF-1/set was demonstrated by yeast two-hybrid technology to interact with NF-protocadherin, which is the *Xenopus* orthologue of protocadherin-7 [34]. Furthermore, mouse protocadherin-18, which is a δ2-protocadherin, has been shown to interact with Disabled-1 [35]. This protein is involved in the Reelin pathway, needed for

correct formation of cortical neuron layers. In terms of the conserved cytoplasmic domains within the δ-protocadherin subfamily (CM1, CM2 and others; data not shown), it would be worthwhile to investigate whether Disabled-1 or TAF-1/set can interact with other members as well.

Interestingly, the gene organization of both δ1- and δ2-protocadherins share a high similarity regarding the positioning of the CM1 and CM2 motifs in separate exons. The last exon of both the *δ1* and *δ2* family members starts with the CM2 motif, while the CM1 motif lies in a smaller upstream exon. This suggests a common origin of at least part of the cytoplasmic domain of the δ-protocadherins. Elaborating the role of the CM1 and CM2 motifs and their interaction partners could thus lead to identification of a common function of these remarkable cell surface molecules.

δ1-Protocadherins are differentially expressed in developing mouse brain

The expression of the δ1-protocadherins mapped in the present work (Pcdh7, Pcdh9, and Pcdh11) is regionally restricted to subsets of neuroanatomical structures, which only partially overlap. This unique and restricted expression pattern for each δ1-protocadherin resembles the general pattern of expression observed for other cadherins in the central nervous system, such as classic cadherins [for a review, see ref. 2], the δ2-protocadherins Pcdh10 (OL-protocadherin) [9] and Pcdh8 [36], and members of the γ-protocadherin subgroup [37, 38]. Examples of similar types of expression patterns are the expression by specific regions and layers of neocortex and hippocampus (figs 4E–P, 5), by amygdala subregions (figs 4I–P, 5A–C), and by specific brain nuclei of the diencephalon and midbrain (figs 4I–P, 5) [for a review, see ref. 2]. These results suggest strongly that the δ1-protocadherins, like other cadherins, play a role in the regionalization and functional differentiation of brain gray matter.

Additionally, it should be noted that a relatively large number of the neuroanatomical structures expressing Pcdh7, Pcdh9, and Pcdh11 belong to the olfactory and limbic systems, suggesting that they might also be markers for subsets of neural circuits, as has been shown for most classic cadherins [for reviews, see refs 2, 4]. Some of these structures exhibit a gender-specific development and differentiation, e.g., the accessory olfactory system, the amygdala, the tuberal hypothalamus, and the globus pallidus. In this context, it is of interest that PCDH11 (PCDHX/Y) was hypothesized to play a role in generating sexual dimorphisms in the human brain [8].

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