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Protocadherin Pcdh2 shows properties similar to, but distinct from, those of classical cadherins

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SUMMARY

Cell adhesion and several other properties of a recently identified cadherin-related protein, protocadherin Pcdh2, were characterized. A chimeric Pcdh2 in which the original cytoplasmic domain was replaced with the cytoplasmic domain of E-cadherin was expressed in mouse L cells. The expressed protein had a molecular mass of about 150 kDa and was localized predominantly at the cell periphery, as was the wild-type Pcdh2. In a conventional cell aggregation assay, the transfectants showed cell aggregation activity comparable to that of classical cadherins. This activity was Ca^{2+}-dependent and was inhibited by the addition of anti-Pcdh2 antibody, indicating that the chimeric Pcdh2, and probably the wild-type Pcdh2, has Ca^{2+}-dependent cell aggregation activity. Mixed cell aggregation assay using L cells and different types of transfectants showed that the activity of Pcdh2 was homophilic and molecular type specific and that Pcdh2 transfectants did not aggregate with other types of transfectants or with L cells. In immunoprecipitation, the chimeric Pcdh2 co-precipitated with a 105 kDa and a 95 kDa protein, whereas wild-type Pcdh2 co-precipitated with no major protein. Pcdh2 was easily solubilized with non-ionic detergent, in contrast to the case of classical cadherins. On immunofluorescence microscopy, the somas of Purkinje cells were diffusely stained with anti-human Pcdh2 antibody. Mouse *Pcdh1* and *Pcdh2* were mapped to a small segment of chromosome 18, suggesting that various protocadherins form a gene cluster at this region. The present results suggest that Pcdh2, and possibly other protocadherins as well as protocadherin-related proteins such as *Drosophila fat*, mediate Ca^{2+}-dependent and specific homophilic cell-cell interaction in vivo and play an important role in cell adhesion, cell recognition, and/or some other basic cell processes.

Key words: cadherin, catenin, cell adhesion, chromosome mapping, protocadherin

INTRODUCTION

Protocadherins are a group of cadherin-related membrane proteins identified recently by using the polymerase chain reaction (PCR) (Sano et al., 1993). In contrast to the typical cadherins, the extracellular domains of protocadherins consist of more than five repeats composed of a unique cadherin motif; the repeats are very similar to each other in size and sequence features, whereas their cytoplasmic domains have no apparent homology with each other or with those of classical cadherins or any other protein sequences in the data bank (Sano et al., 1993). Protocadherins are likely to constitute a large protein family belonging to the cadherin superfamily (for review see Takeichi, 1991; Geiger and Ayalon, 1992; Grunwald, 1993). Similar proteins have been identified also in invertebrates, and are likely to be expressed in different tissues of various organisms (Mahoney et al., 1991; Sano et al., 1993; Oda et al., 1994).

We have recently determined the putative entire coding sequences for two human protocadherins, Pcdh1 and Pcdh2, formerly named pc42 and pc43, respectively, and we have characterized some of their properties. These protocadherins appear to have Ca^{2+}-dependent homophilic interaction, as do classical cadherins, but the aggregation activity of the L cell transfectants is weak, which hampers further characterization of the activity. The cytoplasmic domain of classical cadherins plays an important role in their function (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). This domain can interact with at least three different proteins, α-, β- and γ-catenins (Ozawa et al., 1989). α-catenin, which is homologous to vinculin (Nagafuchi et al., 1991), appears to be necessary for the strong cell aggregation activity of classical cadherins

(Ozawa et al., 1989; Nagafuchi and Takeichi, 1989; Hirano et al., 1992). On the other hand, β-catenin is homologous to plakoglobin (McCrea and Gumbiner, 1991) and γ-catenin appears to be plakoglobin itself (Knudsen and Wheelock, 1992). These proteins, as well as the product of *Drosophila* segment polarity gene *armadillo*, constitute a protein family (Peifer and Wieschaus, 1990), but the role of these proteins in cadherin function is unknown. Since the cytoplasmic domain sequence and the cell adhesion properties of Pcdh1 and Pcdh2 are distinct from those of classical cadherins, it is likely that the cytoplasmic domain of protocadherins may interact with proteins other than catenins. However, no additional information is currently available regarding this possibility.

Protocadherins Pcdh1 and Pcdh2 are highly expressed in brain. Their expression is developmentally regulated, and northern blot analysis showed this expression to be higher in adult brain than in fetal brain (Sano et al., 1993). It is unknown, however, in what cells these protocadherins are primarily expressed in brain and where they are localized in the cells. The answers to these questions may provide a clue to the biological role of these protocadherins.

The genes of classical cadherins form several clusters on different chromosomes (Eistetter et al., 1988; Walsh et al., 1990; Hatta et al., 1991; Kaupmann et al., 1992; Miyatani et al., 1992; Matsunami et al., 1993). Since classical cadherins and protocadherins appear to be derived from the same primordial protein, and since protocadherins constitute a large protein family (Sano et al., 1993), protocadherin genes may also form a cluster (or clusters) on a specific chromosome(s). Information on the chromosomal location of protocadherins may reveal the relationship between classical cadherins and protocadherins as well as a possible connection between protocadherins and some diseases.

As noted above, protocadherins have been shown to possess some unique features, but many questions remain to be answered. Accordingly, we examined some properties of protocadherins, especially Pcdh2, in order to obtain more information that may help to elucidate their biological role. The results show that Pcdh2 has properties similar to, but distinct from, those of classical cadherins. Here we describe the results and discuss their possible biological implications.

MATERIALS AND METHODS

Antibodies

Rabbit anti-human Pcdh2 antibody was prepared as follows. The cDNA that corresponds to the region from the beginning of EC-1 through the end of EC-3 of human Pcdh2 was synthesized by PCR. The primers used contain *Eco*RI or *Xba*I sequence at the 5′ end. The resultant cDNA was digested with *Eco*RI and *Xba*I and subcloned into the *Eco*RI-*Xba*I site of an expression vector, pMal-cRI, and *Escherichia coli* NM522 cells were transformed with the construct. The fusion protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) and was purified from the cell extract by amylose resin column chromatography, as described by the manufacturer. The resultant fusion protein was used without further purification for immunization of a rabbit. The preparation of monoclonal anti-human Pcdh2 antibody (38I2C) was described previously (Sano et al., 1993). Monoclonal anti-mouse α-catenin was a gift from Dr Sh. Tsukita. Monoclonal anti-mouse β-catenin was obtained from Transduction Laboratories (Lexington, KY). Alkaline phosphatase-conjugated anti-rabbit, anti-rat and anti-mouse antibodies were obtained from Promega (Madison, WI). FITC-conjugated anti-rabbit, anti-rat and anti-mouse antibodies were purchased from Cappel (Durham, NC).

Construction of chimeric Pcdh2 cDNA with the cytoplasmic domain sequence of E-cadherin and transfection of L cells

The Bluescript SK plasmid DNA that contains the entire coding sequence for Pcdh2 was digested with *Eco*RV and then partially digested with *Xba*I to remove the sequence that corresponds to the cytoplasmic domain, and the plasmid DNA was purified by agarose gel electrophoresis. The cDNA that corresponds to the cytoplasmic domain of mouse E-cadherin was synthesized by PCR, using as a template mouse cDNA made from mouse lung mRNA and the following two primers: 5′-TCTAGAACGGTGGTCAAAGAGCC and 5′-AGACTTGCTAGTCCCCTA. These primers correspond to the sequence near the N terminus of the cytoplasmic domain and the sequence that contains the stop codon of mouse E-cadherin (Nagafuchi et al., 1987), respectively. PCR was carried out as described previously (Suzuki et al., 1991). *Xba*I sequence was introduced to the 5′ end of the upstream primer for subsequent subcloning of the cDNA. The E-cadherin cDNA was then subcloned into the *Xba*I-*Eco*RV site of the above plasmid DNA. The DNA containing the entire coding sequence was then cut out with *Spe*I and *Eco*RV and subcloned into the *Spe*I-blunted *Xba*I site of an expression vector, pRc/RSV. Finally, L cells were transfected with the resultant construct by a calcium phosphate method using a Pharmacia CellPhect kit. After screening with G418 for about 10 days, the transfectants were stained with FITC-labeled anti-Pcdh2 antibody and subjected to fluorescence activated cell sorting. A number of highly labeled cells were isolated and cloned.

Cell aggregation assay

Cell aggregation activity of the transfectants was assayed as described in a previous report (Sano et al., 1993). Antibody inhibition of cell aggregation was examined by incubation of the transfectants in the standard assay medium, to which anti-Pcdh2 antibody (100 ng/ml) had been added.

Specificity of cell aggregation was determined by the method described by Hatta et al. (1988) with some modifications. The Pcdh2 transfectants or chimeric Pcdh2 transfectants were labeled with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) for 20 minutes at room temperature. After washing with phosphate-buffered saline (PBS), the resultant cells were trypsinized in the presence of 1 mM EGTA, and a single cell suspension was made. The cells were then mixed with unlabeled transfectants of other types and incubated on a rotary shaker for up to two hours, after which they were examined by fluorescence and phase contrast microscopy. As described previously (Sano et al., 1993), Hepes-buffered saline was used for short time aggregation assays (1 hour), and Hepes-buffered saline-Dulbecco's modified Eagle's medium (DMEM) (1:1) mixture was used for longer aggregation assays (1-2 hours).

Immunoprecipitation

The transfectants were metabolically labeled by incubation overnight in DMEM containing [^{35}S]methionine (50 μCi/ml). After washing, the transfectants were solubilized with PBS containing Triton X-100 and NP-40 (Ozawa et al., 1989) and incubated with anti-Pcdh2 antibody. The immunocomplexes were then collected by Protein A-Sepharose, washed with PBS, separated by SDS-PAGE and subjected to autoradiography (Tanihara et al., 1994).

Chromosome localization

Chromosome locations of protocadherins were determined using C3H/HeJ-*gld* and *Mus spretus* (Spain) mice and [(C3H/HeJ-*gld* × *Mus spretus*) F1 × C3H/HeJ-*gld*] interspecific backcross mice as previously described (Seldin et al., 1988). *Mus spretus* was chosen as the

second parent in this cross because of the relative ease of detection of informative restriction fragment length variants (RFLV) in comparison with crosses using conventional inbred laboratory strains. For protocadherins *Pcdh1* and *Pcdh2*, rat cDNA clones corresponding to respective EC3-EC4 were used.

For fibroblast growth factor a (*Fgf1*), the previously described clone (pJC3-5) was used as a probe (Jaye et al., 1986). Informative *Fgf1* RFLVs were detected using *Bam*HI restricted DNA (C3H-*gld*, 7.2 kb; *Mus spretus* 12.0 kb).

Gene linkage was determined by segregation analysis (Green, 1981). Gene order was determined by analyzing all haplotypes and minimizing crossover frequency between all genes that were determined to be within a linkage group (Bishop, 1985).

Other procedures

Northern blot analysis was performed according to the method of Thomas (1980). Western blot analysis and immunofluorescence microscopy were carried out as described previously (Sano et al., 1993). Cells were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere.

RESULTS

Expression of chimeric protocadherin Pcdh2 with cytoplasmic domain of E-cadherin

L cell transfectants of Pcdh1 and Pcdh2 showed only weak cell aggregation activity, which hampered the detailed characterization of this activity. To further characterize the properties, we constructed a chimeric Pcdh2 based on the following assumption: the weak cell aggregation activity of Pcdh2 in L cells may be attributable to the unique cytoplasmic domain, which has no apparent homology with that of classical cadherins, whereas the extracellular domain may retain the ability of strong homophilic interaction, because it contains features common to the cadherin extracellular domain motif. If this is the case, the chimeric Pcdh2 with the cytoplasmic domain of a classical cadherin may show stronger homophilic interaction than that of wild-type Pcdh2, which would permit further characterization of the protocadherin interaction.

The cDNA for the cytoplasmic domain of mouse E-cadherin was synthesized by PCR using the published sequence (Nagafuchi et al., 1987), and the cDNA sequence for the cytoplasmic domain of protocadherin Pcdh2 was replaced with that of E-cadherin (Pcdh2/E). The construct was then introduced into L cells by a calcium phosphate method and the chimeric protocadherin was expressed. The expressed protein consisted of two adjacent bands of about 150 kDa in molecular mass (Fig. 1), which is slightly larger than that of wild-type Pcdh2. The chimeric protein was very labile and easily digested with trypsin. As shown for wild-type Pcdh2 (Sano et al., 1993), the chimeric protein was not protected from the trypsin digestion with Ca^{2+}. The transfectants showed a morphology very similar to that of parental L cells (Fig. 2) and the expressed protein was localized predominantly at the cell periphery, especially at cell-cell contact sites, which is slightly different from that of wild-type Pcdh2 transfectants; the expressed protein of wild-type Pcdh2 was localized mainly at cell-cell contact sites, but it was also present diffusely on the cell surface. In cell aggregation assay, the transfectants showed clear Ca^{2+}-dependent cell aggregation within 40 minutes of incubation (Fig. 3a and b). Cell aggregation required more than 1 mM of Ca^{2+} in the assay medium and was inhibited by omitting Ca^{2+} from the assay solution (Fig. 3f). The cell adhesion activity was comparable to that of cadherin-4 transfectants (Tanihara et al., 1994). This is in marked contrast to that of wild-type protocadherin Pcdh2 transfectants, which require nearly two hours to aggregate, and the resultant aggregates were much smaller than those of the chimeric transfectants (Sano et al., 1993). The difference in cell aggregation activity is not attributable to a difference in the expression level of protocadherins, since we used transfectants that expressed similar levels of proteins, as shown by western blot analysis. This aggregation was inhibited by the addition of anti-Pcdh2 antibody (Fig. 3e). Furthermore, when L cells labeled with DiO were mixed with unlabeled Pcdh2/E transfectants and subjected to the cell aggregation assay, the transfectants did not co-aggregate significantly with parental L cells (Fig. 3c and d), suggesting that the interaction was homophilic. These results indicate clearly that the extracellular domain of protocadherin Pcdh2 has the capability of significant Ca^{2+}-dependent homophilic interaction.

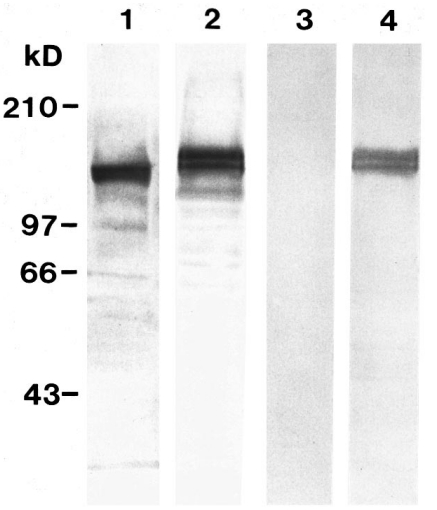

Fig. 1. Immunoblotting of chimeric protocadherin Pcdh2/E. The chimeric cDNA containing E-cadherin cytoplasmic domain sequence instead of the original Pcdh2 cytoplasmic domain sequence was constructed and expressed in mouse L cells. The expressed protein was examined by immunoblotting. Antibody against the extracellular domain of Pcdh2 stained Pcdh2 (lane 1) and Pcdh2/E transfectants (lane 2), while pancadherin antibody stained Pcdh2/E (lane 4) but not Pcdh2 transfectants (lane 3). The expressed Pcdh2/E was slightly larger than the authentic Pcdh2 protein.

Specificity of cell aggregation activity

When Pcdh2 transfectants labeled with DiO were incubated with unlabeled Pcdh1 transfectants in a cell aggregation assay, labeled and unlabeled cells aggregated in an almost mutually exclusive manner (Fig. 4a and d), whereas labeled and unlabeled Pcdh2 formed mixed aggregates (Fig. 4c and f). These results indicate that the interactions of protocadherins have specificity. Because the cell aggregation activities of Pcdh1 and Pcdh2 transfectants were much weaker than were those of classical cadherins, this method could not be applied directly to the examination of the interaction between protocadherins and classical cadherins. Hence, we examined the interaction between the chimeric protocadherin Pcdh2/E and

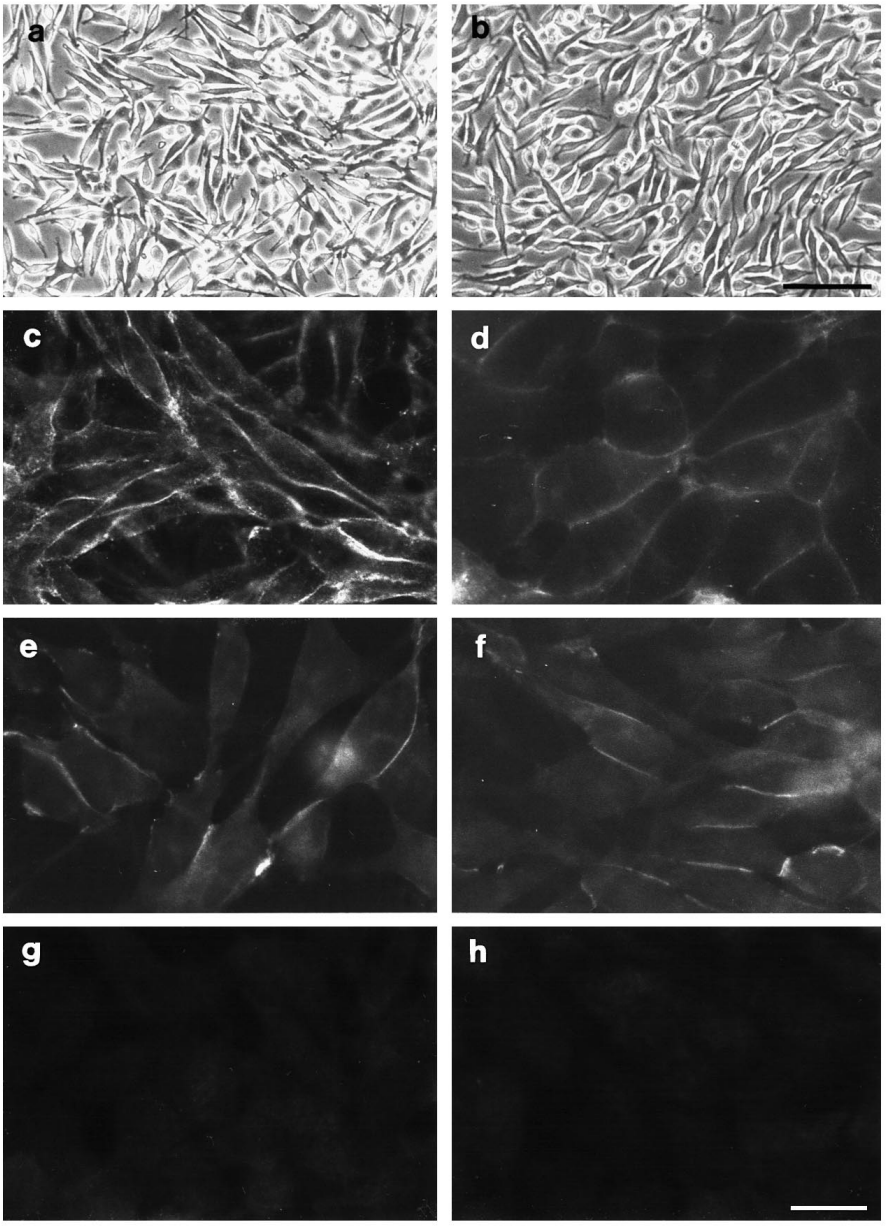

Fig. 2. Morphology and immunofluorescence staining of chimeric protocadherin Pcdh2/E. The Pcdh2/E transfectants (a) showed a morphology very similar to that of parental L cells (b). The chimeric protein was localized at the cell periphery, mainly at cell-cell contact sites, as in wild-type Pcdh2 (c). However, wild-type Pcdh2 was also detected diffusely on the cell surface (d). Anti-α-catenin (e,g) and anti-β-catenin (f,h) antibodies stained similar sites of the Pcdh2/E transfectant (e,f), but not the parental L cells (g,h). Bars: 100 μm (a,b) and 20 μm (c-h).

cadherin-4, since Pcdh2/E showed cell aggregation activity comparable to that of classical cadherins. As shown in Fig. 4b and e, Pcdh2/E transfectants did not display significant aggregation with cadherin-4 transfectants, indicating that Pcdh2/E has specific cell aggregation activity.

Association of protocadherin Pcdh2 and Pcdh2/E with catenins

If protocadherins can affect some intracellular processes, they are certainly mediated via the cytoplasmic domains through interaction with cytoplasmic proteins, as are the classical cadherins. To ascertain if this is the case, immunoprecipitation of wild-type protocadherin Pcdh2 and chimeric Pcdh2/E was carried out. The Pcdh2/E co-precipitated with a 105 kDa and a 95 kDa protein, which presumably correspond to α- and β-catenins, respectively, as does E-cadherin (Fig. 5), since anti-α-catenin and anti-β-catenin antibodies stained the bands comparable to the 105 kDa and the 95 kDa bands in the immunoprecipitated samples of the chimeric Pcdh2/E transfectants. The levels of α-catenin and β-catenin increased in Pcdh2/E clones as compared with those of parental L cells. Pcdh2, on the other hand, did not co-precipitate with any major protein when both L cell transfectants of Pcdh2 and SK-N-SH neuroblastoma cells that express Pcdh2 were used. Furthermore, Pcdh2 was easily extracted with non-ionic detergent and the insoluble fraction of the extraction experiments did not contain a significant amount of Pcdh2, in sharp contrast to the case of classical cadherins (Hirano et al., 1987; Ozawa et al., 1989).

Expression of protocadherin Pcdh2 in cerebellum

Although Pcdh2 is highly expressed in brain, where in brain it is expressed or how its expression is regulated is unknown. As a first attempt to address this question, we carried out in situ hybridization experiments using rat brains. Preliminary results

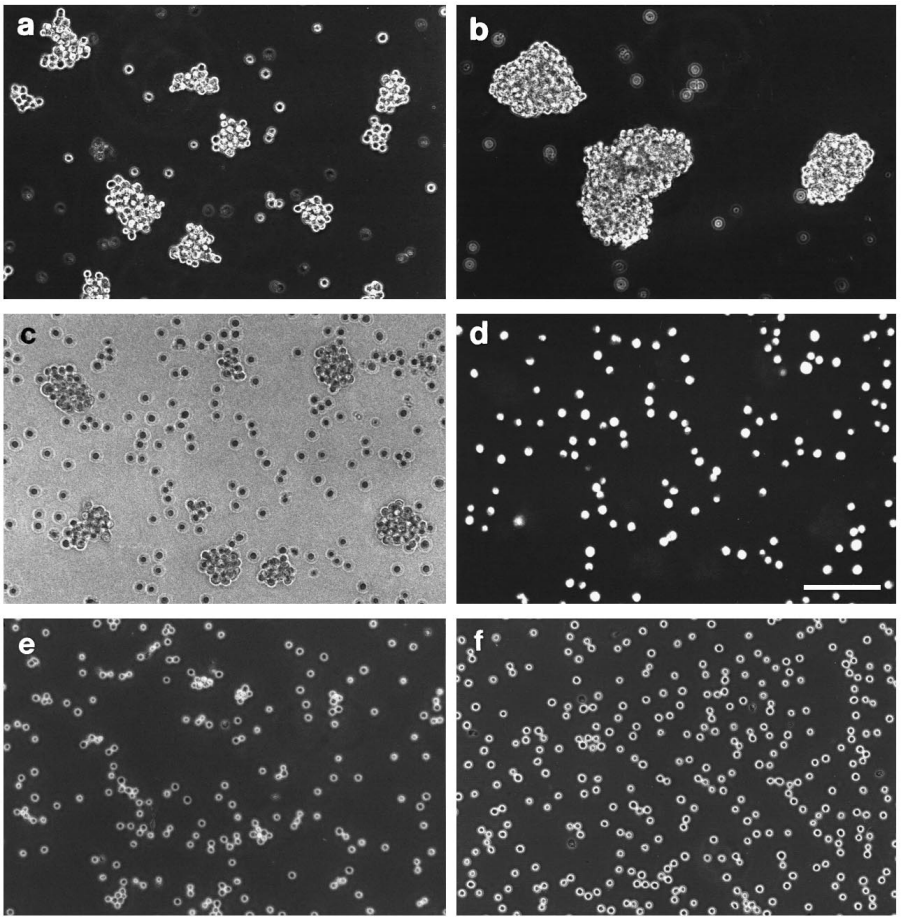

Fig. 3. Cell adhesion activity of Pcdh2/E. The cell adhesion activity of the chimeric transfectants was examined by a cell aggregation assay described by Urushihara et al. (1979), with some modification (Ozawa et al., 1990). The Pcdh2/E transfectants (a) started to aggregate after about 40 minutes of incubation and the aggregates were much larger than those of the wild-type Pcdh2 transfectants and comparable to those of cadherin-4 transfectants (b). The aggregation was inhibited by the addition of anti-Pcdh2 antibody (e) or by omitting Ca^{2+} (f) in the cell aggregation assay solution. When L cells labeled with DiO were mixed with Pcdh2/E transfectant cells and subjected to the cell aggregation assay, Pcdh2/E transfectants aggregated almost exlusively (c,d), suggesting that the interaction is homophilic. (c) Phase contrast micrograph; (d) immunofluorescence micrograph. Bar, 50 μm.

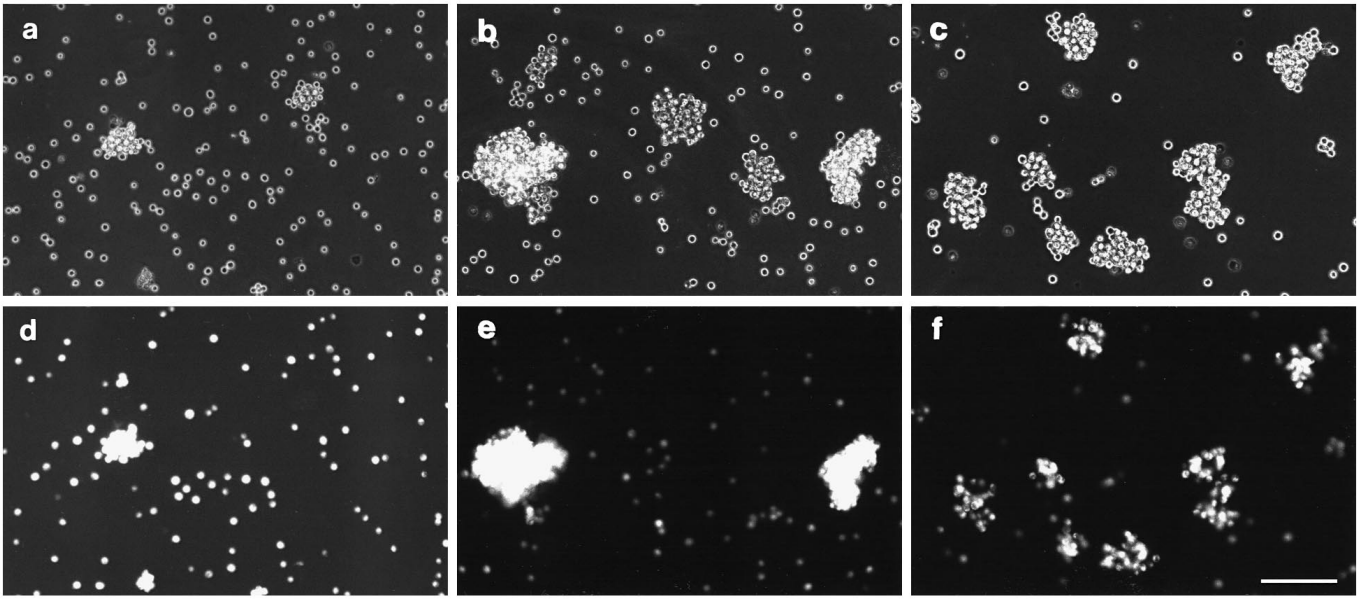

Fig. 4. Specificity of cell adhesion activity of Pcdh2. The Pcdh2 transfectants were labeled with DiO and mixed with unlabeled Pcdh1 transfectants and incubated on a rotary shaker for 2 hours. The labeled Pcdh2 transfectants and unlabeled Pcdh1 transfectants were aggregated almost mutually exclusively (a,d). Similarly, DiO labeled cadherin-4 transfectants and unlabeled transfectants of the chimeric protocadherin Pcdh2/E were mixed and incubated on a rotary shaker. After one hour of incubation, both transfectants formed relatively large aggregates, but each transfectant aggregated almost exclusively with the same type of transfectant (b,e). When Pcdh2/E transfectants labeled with DiO and the unlabeled Pcdh2/E transfectants were mixed and subjected to the cell aggregation assay, labeled and unlabeled cells formed mixed cell aggregates (c,f). (a-c) Phase contrast micrographs; (d-f) fluorescence micrographs. Bar, 50 μm.

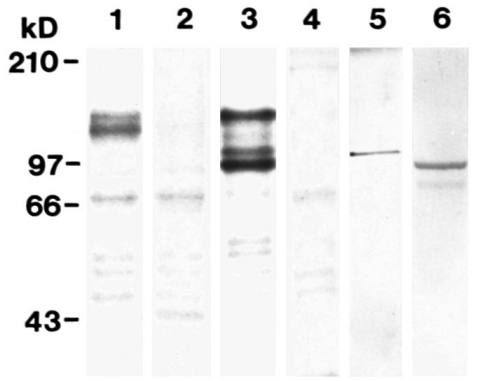

Fig. 5. Immunoprecipitation of Pcdh2 and Pcdh2/E. The chimeric Pcdh2/E co-precipitated with a 105 kDa and a 95 kDa band (lane 3), whereas the wild-type Pcdh2 co-precipitated with no major band (lane 1). Anti-α-catenin (lane 5) and anti-β-catenin (lane 6) antibodies stained corresponding bands in immunoprecipitated samples. Myosin, phosphorylase b, albumin and ovalbumin were used as size markers. Lanes 2 and 4, parental L cells.

indicated that the Pcdh2 probe detected a strong signal in Purkinje cells of rat cerebellum (N. Mori et al., unpublished observation). On immunofluorescence microscopy, the somas of human Purkinje cells were stained with anti-Pcdh2 antibodies, whereas the axons of Purkinje cells were devoid of the staining. The punctate artifact staining near Purkinje cells appears to be due to erythrocytes, since the staining corresponded to erythrocytes on phase-contrast microscopy. Furthermore, western blot analysis showed a 150 kDa band in human cerebellum, which is indistinguishable from that of SK-N-SH neuroblastoma cells and one of the bands of Pcdh2 transfectants (Fig. 6). These results indicate that Pcdh2 is expressed on the cell membrane of soma of Purkinje cells.

Chromosome mapping of Pcdh1 and Pcdh2

In order to determine the chromosomal location of the various protocadherins, we analyzed a panel of DNA samples from an interspecific cross that has been characterized for over 800 genetic markers throughout the genome. The genetic markers included in this map span between 50 and 80 centi-Morgans (cMs) on each mouse autosome and the X chromosome (for example, see Saunders and Seldin, 1990; Watson et al., 1992). Initially, DNAs from the two parental mice [C3H/HeJ-*gld* and (C3H/HeJ-*gld* × *Mus spretus*)F1] were digested with various restriction endonucleases and hybridized with the cDNA probes to determine RFLVs to allow haplotype analyses. Informative RFLVs for each clone are listed in Table 1.

Table 1. Restriction fragment length variants of protocadherin *Pcdh1* and *Pcdh2*

Locus	Restriction endonuclease	Fragment size (kbp)	
		C3H-*gld*	*Mus spretus*
Pcdh1	*Taq*I	1.2	0.7
Pcdh2	*Eco*RI	6.2	16.0

Comparison of the haplotype distribution of mouse *Pcdh1* and *Pcdh2* genes with those determined for loci throughout the mouse genome allowed each to be mapped to a specific region of mouse chromosome 18 (Fig. 7) (Davisson and Johnson, 1992). The probability for linkage was >99%, and the best gene order (Bishop, 1985) ± the standard deviation (Green, 1981) indicated the following relationship: (centromere)–*Pcdh1*/*Pcdh2*–0.9 cM±0.9 cM–*Fgf1*–0.9±0.9 cM–*Grl1* (Fig. 8).

DISCUSSION

This study has revealed some properties of protocadherin Pcdh2 that have several interesting implications for the biological role of protocadherins. The experiments on chimeric protocadherin Pcdh2/E clearly indicate that the extracellular domain of Pcdh2 is capable of Ca^{2+}-dependent and specific homophilic interaction, as was shown for classical cadherins. We have not performed similar experiments with Pcdh1 or

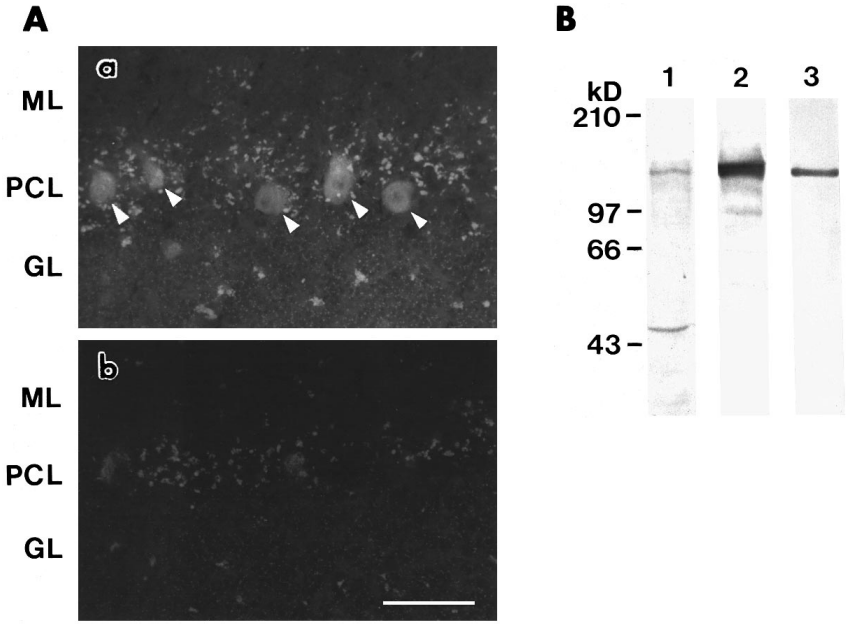

Fig. 6. Expression of Pcdh2 in Purkinje cells. (A) Anti-Pcdh2 antibody diffusely stained the cell membrane of large Purkinje cell body (arrowheads), but not the axons in immunofluorescence microscopy (a). The staining without the first reaction with anti-Pcdh2 antibody showed no significant signal in Purkinje cells (b). GL, granular cell layer; ML, molecular cell layer; PCL, Purkinje cell layer. Bar, 50 μm. (B) In western blot analysis of human cerebellum, the anti-human Pcdh2 antibody stained a 150 kDa band (lane 1) that is indistinguishable from those obtained from Pcdh2 transfectant cells (lane 2) and SK-N-SH human neuroblastoma cells (lane 3). The size markers used were the same as described in Fig. 5.

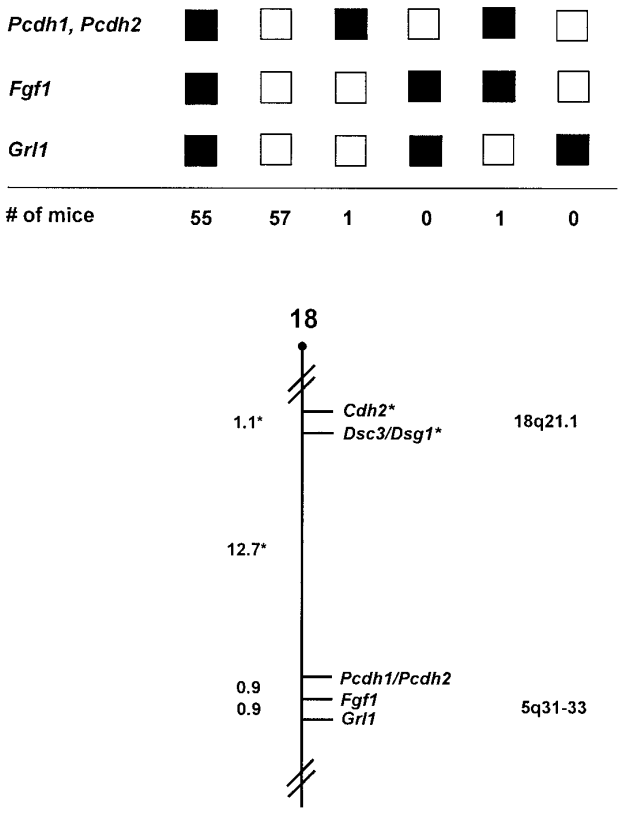

Fig. 7. Chromosome localization of mouse *Pcdh1* and *Pcdh2*. (A) The segregation of *Pcdh1* and *Pcdh2* among mouse chromosome 18 loci in [(C3H/HeJ-*gld* × *Mus spretus*)F1 × C3H/HeJ-*gld*] interspecific backcross mice is shown. The loci are listed from proximal to distal on the left side. Each column represents a possible haplotype and the number of mice observed with each haplotype is indicated at the bottom of the column. The boxes indicate whether the mice were typed as C3H/HeJ-*gld* homozygotes (filled) or F1 heterozygotes (open) for each locus. (B) Partial linkage map of mouse chromosome 18 is shown. The locations of N-cadherin (*Cdh2*), desmocollin 3 (*Dsc3*) and desmoglein 1 (*Dsg1*) are from Miyatani et al. (1992) and Buxton et al. (1994), respectively (*). Recombination distances between loci are shown in centi-Morgans at the left side of the chromosome. The corresponding locations in human chromosomes are shown to the right.

other protocadherins, but it is highly likely that the extracellular domains of other protocadherins have similar cell adhesion properties, considering their structural similarity (Sano et al., 1993). Oda et al. (1994) recently reported a cadherin-related protein from *Drosophila*. Most of the extracellular domain of this protein consists of more than five repeats of a cadherin motif that is very similar to that of protocadherin extracellular domain, whereas the cytoplasmic domain has significant homology with that of classical cadherins. Interestingly, transfectants of this protein showed cell adhesion properties very similar to those of classical cadherins. Their results are thus consistent with ours. *Drosophila fat* is a tumor suppresor gene and its function appears to be mediated through cell-cell interaction (Mahoney et al., 1991). The extracellular domain motif of *Drosophila fat* is essentially the same as those of the protocadherins and the cadherin-related *Drosophila* protein (Mahoney et al., 1991; Sano et al., 1993; Oda et al., 1994). On the other hand, Shapiro et al. (1995) recently proposed a hypothesis, deduced from a structural study, that cadherin repeats mediate homophilic interaction. Taken together, it seems possible that the product of *Drosophila fat* is capable of Ca^{2+}-dependent homophilic interaction, and that this interaction may play an important role in its function.

The present results show that the cell adhesion properties of Pcdh2 are very similar to those of classical cadherins, but the cell adhesion activity of Pcdh2 is much weaker than that of classical cadherins. One may argue that the weak activity is due to the lack of necessary components for strong cell adhesion activity in L cells. Many fibroblasts, however, express endogenous Pcdh2, although the levels are relatively low. Therefore, L cells are likely to contain necessary components for Pcdh2 function. Furthermore, Pcdh2 transfectants of mouse neuroblastoma neuro-2a cells did not show strong cell adhesion activity. Thus, the weak cell adhesion activity of Pcdh2 appears to be an intrinsic feature. In immunoprecipitation experiments Pcdh2/E co-precipitated with a 105 kDa and a 95 kDa protein, which appear to correspond to α-catenin and β-catenin, respectively. In contrast, wild-type Pcdh2 did not co-precipitate with these proteins or with any other major proteins in L cell transfectants. These results may explain the difference in cell aggregation activity between the chimeric Pcdh2/E and the wild-type Pcdh2. Stable interaction between the cytoplasmic domain and the cytoplasmic proteins, especially α-catenin, is thought to be essential for the strong cell aggregation activity of classical cadherins (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989; Hirano et al., 1992). Wild-type Pcdh2, on the other hand, cannot associate strongly with cytoplasmic proteins and cannot support strong cell adhesion. Consistent with our conclusion, Shapiro et al. (1995) have recently proposed a hypothesis that cadherin repeats have intrinsically weak homophilic interaction activity and the relatively strong cell adhesion activity of classical cadherins can be explained by a special property of classical cadherins.

Although protocadherin Pcdh2 can mediate homophilic interaction, Pcdh2 appears to be localized diffusely on the cell surface of Purkinje cells, but the surrounding cells did not show significant expression of Pcdh2. This is not Purkinje cell-specific distribution as we recently found that syncytiotrophoblasts of human placenta express high levels of Pcdh2, localized on the entire cell surface but especially on apical surface villi (S. Obata et al., unpublished observation). If Pcdh2 is involved in some type of cell-cell interaction, these results imply that Pcdh2 also has heterophilic interaction activity. Indeed, several groups have already suggested that a classical cadherin, N-cadherin, may also exhibit heterophilic interaction in addition to homophilic interaction (Salomon et al., 1992; Cifuentes-Diaz et al., 1994). Furthermore, Cepek et al. (1994) recently showed evidence that E-cadherin can interact with an integrin. Since this issue is important for understanding the biological role of protocadherins as well as classical cadherins, further studies are required.

The present results show that the homophilic interaction of protocadherin Pcdh2 (and perhaps Pcdh1) and other protocadherins has specificity, as do classical cadherins. Given the large number of protocadherins expressed in brain, our results further suggest that various specific cell-cell interactions can be mediated by protocadherins in brain. Furthermore, if protocadherins can also mediate heterophilic interaction, as

discussed above, the potential variability of the interactions may be rather complex. The central nervous system requires a variety of cell-cell interactions to form and maintain the tissue structure and to fulfill its complex function. Indeed, many cell adhesion proteins have recently been isolated from the central nervous system. Thus, it is very tempting to speculate that protocadherins may be involved in the complex cell-cell interactions in the central nervous system.

Classical cadherins and protocadherins are thought to be derived from the same primordal protein, but the chromosomal locations of classical cadherins and protocadherins are different (Eistetter et al., 1988; Walsh et al., 1990; Hatta et al., 1991; Kaupmann et al., 1992; Miyatani et al., 1992; Matsunami et al., 1993). Most of the classical cadherins are on mouse chromosome 8, whereas Pcdh1 and Pcdh2 are mapped to a specific region of mouse chromosome 18. The close linkage of Pcdh1 and Pcdh2 suggests that multiple protocadherins form a gene cluster at this region, much as various classical cadherins do. Actually, we have recently obtained evidence that at least two additional protocadherin genes are present in this region (S. Obata et al., unpublished observation). Interestingly, one classical cadherin, N-cadherin, and two desmosomal cadherins are mapped to a region near the protocadherin loci on mouse chromosome 18 (Miyatani et al., 1992; Buxton et al., 1994), but it is unclear whether this has evolutional relevance.

As described above, protocadherins appear to form a gene cluster in a specific region on mouse chromosome 18. This region corresponds to that of the ataxia (*ax*) and twirler (*Tw*) genes, the mutations that affect some neural activity (Lyon, 1955, 1958; Burt, 1980). Therefore, it seems possible that one of the protocadherins corresponds to the ataxia or twirler gene. The present results indicated that Pcdh2 was expressed in cerebellum and that this expression was developmentally regulated. Since available information suggests that protocadherins are involved in some processes of the central nervous system, it is possible that mutation of these genes may result in some neural diseases. Further studies are necessary to clarify the biological role of protocadherins in brain and other tissues. However, results of the present study indicate that protocadherins have properties both similar to and unique from those of classical cadherins and suggest their involvement in some important processes in the brain and other tissues.

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