Interaction with Protocadherin-γ Regulates the Cell Surface Expression of Protocadherin-α*

Yoji Murata‡§, Shun Hamada‡, Hirofumi Morishita‡, Tetsuji Mutoh‡¶, and Takeshi Yagi‡§**

From the §KOKORO Biology Group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, 565-0871, the ‡Laboratory of Neurobiology and Behavioral Genetics, National Institute for Physiological Sciences, Okazaki 444-8585, and ¶Core Research for Evolutional Science and Technology of the Japan Science and Technology Agency, Japan

The protocadherin-α (CNR/Pcdha) and protocadherin-γ (Pcdhγ) proteins, members of the cadherin superfamily, are putative cell recognition/adhesion molecules in the brain. Overexpressed cadherins are generally expressed on the cell surface and elicit cell adhesion activity in several cell lines, although hardly any overexpressed CNR/Pcdha proteins are expressed on the cell surface, except on HEK293T cells, which show low expression. We analyzed the expression of CNR/Pcdha and Pcdhγ in HEK293T cells and found that they formed a protein complex and that Pcdhγ enhanced the surface expression of CNR/Pcdha. This enhanced surface expression was confirmed by flow cytometry analysis and by marking cell surface proteins with biotin. The enhancement was observed using different combinations of CNR/Pdho and Pcdhγ proteins. The surface expression activity was enhanced by the extracellular domains of the proteins, which could bind each other. Their cytoplasmic domains also had binding activity and influenced their localization. Their protein-protein interaction was also detected in extracts of mouse brain and two neuroblastoma cell lines. Thus, interactions between CNR/Pdha and Pcdhγ regulate their surface expression and contribute to the combinatorial diversity of cell recognition proteins in the brain.

Cell recognition molecules play significant roles in the building of neuronal networks in the central nervous system. The cadherin superfamily is a large family of calcium-dependent cell adhesion molecules, which have been implicated in the morphogenesis of nonneuronal and neuronal tissues (1). Classical cadherins have five tandemly repeated extracellular (EC) domains that mediate calcium-dependent homophilic protein interactions (2). Overexpressed classic cadherins are generally expressed on the cell surface and elicit cell adhesion activity in several cell lines. The cell adhesion activity of the classic cadherins plays an important role in neuronal development, including the formation and maintenance of neuronal connectivity and synaptic plasticity (3–8).

The protocadherins (Pdhs) and cadherin-related neuronal receptor (CNR) are putative trans-synaptic recognition molecules that have six EC domains and a cytoplasmic domain (9, 10). Genes for the CNR/Pdhs are organized into clusters (termed α (CNR), β, and γ (11, 12)). Within the α and γ clusters, three exons encode the cytoplasmic domain for each Pcdh, making these domains identical within a cluster. The extracellular regions of Pcdh proteins have been shown to mediate weak, homophilic cell adhesion (13). The CNR/Pcdha proteins appear not to possess homophilic-binding activity, whereas CNR/Pdho-v4 becomes a calcium-dependent cell adhesion molecule upon interacting with integrins. The interacting site is an RGD motif in the EC1 region, which is highly conserved among mammalian CNR/Pdho members (14).

We previously transfected full-length CNR/Pdha-v4 into several cell lines and did not detect the overexpressed protein on the cell surface of L1, Neuro2A, COS7, Chinese hamster ovary, CHP212, or MDCKII cells. In these cell lines, the CNR/Pdha-v4 was concentrated in the endoplasmic reticulum. However, in HEK293T cells, low levels of CNR/Pdha-v4 were detected on the cell surface, although most remained in the endoplasmic reticulum. These results indicated that in the cell lines tested, almost no CNR/Pdha protein is transported to the cell surface, except for HEK293T cells, where there is a low level of surface expression (14). The immunolocalization of Pcdhγ in hippocampal neurons showed it to be strongly expressed on a subpopulation of tubulovesicular structures and to be recruited to the synapse in Pcdhγ-positive neuron pairs (15). These results suggested that the CNR/Pdha and Pcdhγ proteins have molecular features that enable the regulation of their expression on the surface of the synaptic plasma membrane from intracellular compartments.

CNR/Pdha and Pcdhγ are widely expressed in the brain. Single cell analyses using reverse transcription-PCR and in situ hybridization have revealed that each neuronal cell expresses distinct sets of CNR/Pdha and Pcdhγ proteins (10, 16, 17). CNR/Pdha and Pcdhγ proteins are commonly concentrated in the post-synaptic density fraction. The biochemical and functional interactions of CNR/Pdha and Pcdhγ, however, remain unknown. Here we show that CNR/Pdha and Pcdhγ formed a protein complex in the brain and neuroblastoma. They bound each other at sites in both their extracellular and cytoplasmic domains. Formation of the complex enhanced the cell surface expression of the CNR/Pdha proteins. Thus CNR/

* This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (to T. Y.), the Uehara Memorial Foundation, the Takeda Foundation, and CREST (Core Research for Evolutional Science and Technology) of JST (Japan Science and Technology Agency). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Tel.: 81-6-6879-7990; Fax: 81-6-6877-1922; E-mail: yagi@fbs.osaka-u.ac.jp.

† Present address: Laboratory for Cell Culture Development, Brain Science Institute, RIKEN, Saitama 351-0198, Japan.

‡ To whom correspondence should be addressed: Tel.: 81-6-6879-7990; Fax: 81-6-6877-1922; E-mail: yagi@fbs.osaka-u.ac.jp.

§ The abbreviations used are: EC, extracellular domain; Pdho, protocadherin; CNR, cadherin-related neuronal receptor (mouse protocadherin-γ); CP, cytoplasmic domain; PBS, phosphate-buffered saline; NHS, hydroxyysulfosuccinimide; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; aa, amino acid(s); CNRA, CNR alternative splicing form type A; CLCR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein.
Pcdo and Pdhy proteins form a hetero-protein complex, which plays a role in the regulation of protein localization to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Full-length mouse Pdgf-α2, -α3, -α12, -b1, -b2, and -b4 were amplified using reverse transcription-PCR and subcloned into pcDNA3.1. Full-length mouse Cnr/Pcdh-a4 was tagged with Pyc by PCR with the c-myc epitope tag (EQKLISEEDL), recognized by the 9E10 mouse monoclonal antibody. The tag was introduced after amino acid 32 of the nascent Cnr/Pcdh-a4 protein. The Cnr/Pcdh and Pdhy deletion mutants were generated by PCR from these full-length and tagged cDNAs. Myc-Cnr/Pcdh-a4delCP (aa 1–724) and delEC (aa 681–947) were generated by PCR reactions from full-length Cnr/Pcdh-a4 and -a3, -b1, and -b12delCP. Myc were generated by fusing the Myc epitope tag to the carboxyl terminus of Pcdh-a3 (aa 1–712), -a12 (aa 1–717), and -b1 (aa 1–702), respectively. Pcdh-a12EC-His and Pdhy-b12EC-His were generated by fusing His to the extracellular domain of Pcdh-a12 (aa 1–688) and -b1 (aa 1–675). Cnr/Pcdh-a4EC-Myc and Cnr/Pcdh-a7EC-Myc were generated by fusing the Myc epitope tag to the extracellular domains of Cnr/Pcdh-a4 and -a7 (aa 809–932), respectively. Cnr/Pcdh-a4EC-Myc was generated by subcloning the cytoplasmic region of Cnr/Pcdh-a4 (aa 722–948) into pGEX-5X-1 (Amerham Biosciences). The identity of all the constructs was confirmed by dyeoxyducleotide sequencing.

**Cell Culture and Transfection**—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. C1300 and NB2a cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum. C1300 and NB2a cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum. Transfection of plasmid DNA serum. C1300 and NB2a cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum. C1300 and NB2a cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum. Myc antibodies (1

**Immunoprecipitation**—HEK293T cells were transfected with Pcdh-α12, -b2, -a12-delCP-Myc or -b2-delCP-Myc. The transfected cells were lysed with Buffer B containing 1 mM EDTA. The lysates were spun at 20,000 × g for 20 min. The supernatant (S3) was used for immunoprecipitation. The supernatant was incubated with 1 μg of anti-CNR-α, anti-CNR-β, anti-Pcdh-α12, or anti-Pcdh-α12 delCP-Myc for 1 h at 4 °C. The beads were washed extensively with Buffer B. The proteins bound to the beads were analyzed by SDS-PAGE followed by Western blotting. Cnr/Pcdh-a4EC-Myc, Pcdh-α12EC-His, Pdhy-b12EC-His, both Cnr/Pcdh-a4EC-Myc and Pcdh-α12EC-His, or both Cnr/Pcdh-a4EC-Myc and Pdhy-b12EC-His were transinfected into HEK293T cells. Opti-Mem (Invitrogen) supernatants were collected 48 h after transfection. ProBond Resin nickel beads (Invitrogen) or Protein G-Sepharose beads immobilized anti-Myc antibodies (1 μg) were incubated with the supernatants for 1 h at 4 °C. The beads were extensively washed with Buffer C (20 mM Tris-HCl, pH 8.0, 40 mM imidazole, 0.3 mM NaCl, 0.01% Triton X-100) for nickel beads or Buffer D (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% Triton X-100) for Protein G-Sepharose beads. The beads were washed extensively with Buffer D, and the beads were incubated with 5% bovine serum albumin and 1 μM EDTA. Detached cells (1 × 10^6) were incubated with anti-Myc antibodies diluted in PBS containing 0.5% bovine serum albumin for 30 min. The beads were then washed with PBS and incubated with an anti-mouse IgG- alkaline phosphatase (1:500) for 1 h at 4 °C. After extensive washing, the expression of the tagged proteins was quantified using an EPICS Altra (Beckman Coulter).

**Biotinylation of Cell Surface Proteins**—Transfected HEK293T cells were washed with ice-cold PBS three times and incubated 30 min with 50 mM EZ-link sulfo-hydroxysuccinimidyl (NHS)-S-biotin (Pierce) in cold PBS. After washing with ice-cold PBS three times, the cells were lysed with radioimmune precipitation assay buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and spun at 20,000 × g for 30 min. Streptavidin-agarose beads (Pierce) were added to the supernatants, and the mixture was incubated at 4 °C for 1 h. After the beads were extensively washed with radioimmune precipitation assay buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer and subjected to SDS-PAGE followed by Western blot analysis.

**Subcellular Fractionation of Mouse Brain**—Subcellular fractionation of the mouse brain was performed as described previously (19, 20). Briefly, adult mouse brains were homogenized in an ice-cold solution containing 0.32 mM sucrose, 1 mM HEPES-KOH, pH 7.4, 1 mM NaHCO3, 1 mM MgCl2, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aproli-

**Immunohistochemistry**—ICR mice (postnatal days 10–14) were used for the immunohistochemical detection of Cnr/Pcdh and Pdhy proteins. Mice were deeply anesthetized and decapitated. The brains were rapidly removed and immersed in embedding material (OCT compound, Miles). The brains were quickly frozen by isopentane cooled

**RESULTS**

**Localization of CNR/Pdha and Pdhy Proteins**—CNR/Pdha family proteins are localized, in part, to synaptic regions (10), and the Pdhy proteins are targeted to subsets of synapses and intracellular organelles in the hippocampal neurons (15). To address the protein localization of both Cnr/Pdha and
Pedhγ family proteins, we produced affinity-purified antibodies to the constant cytoplasmic domain of CNR/Pedhα (anti-CNR/Pedhα) and Pedhγ (anti-Pedhγ). The variable exons are cis-spliced to the common exon of the CNR/Pedhα or Pedhγ transcript in each gene cluster; therefore, the antibodies should mark the regions in which any of the 14 CNR/Pedhα or 25 Pedhγ proteins is localized, respectively. We tested the antibodies for specificity against lysates from HEK293T cells transfected with mouse CNR/Pedhα and Pedhγ proteins. The anti-CNR/Pedhα and anti-Pedhγ antibodies reacted specifically with the transfected CNR/Pedhα and Pedhγ proteins (data not shown).

We next evaluated the distribution of the CNR/Pedhα and Pedhγ proteins in cultured hippocampal neurons. The CNR/Pedhα and Pedhγ staining occurred as fine puncta, and their expression patterns were partially colocalized (Fig. 1A). A similar distribution of Pedhγ was reported for cultured hippocampal neurons (15). Using adult brains, subcellular fractionation analysis showed both CNR/Pedhα and Pedhγ to be concentrated in the synaptic membrane and post-synaptic density fractions (Fig. 1B). These results suggested that CNR/Pedhα and Pedhγ are colocalized in the brain. To elucidate their colocalization in the hippocampus, we performed immunostaining with the anti-CNR/Pedhα and anti-Pedhγ antibodies. The CNR/Pedhα and Pedhγ proteins were both detected in the molecular layers of the dentate gyrus, CA1, and CA3 regions in the hippocampus (data not shown). At high magnification, the signals of the CNR/Pedhα and Pedhγ proteins showed similar punctate patterns (Fig. 1C). Double staining analysis revealed some double-stained puncta, in addition to the single-stained puncta (Fig. 1B). That is, the patterns of the puncta stained for CNR/Pedhα and Pedhγ, respectively, were not identical, but partially overlapped.

The staining pattern and subcellular fractionation of the CNR/Pedhα and Pedhγ proteins suggested that they might form a complex in vivo. To investigate this possibility, we performed immunoprecipitation analyses of the CNR/Pedhα and Pedhγ proteins. Lysates from mouse brains at postnatal day 7 were subjected to immunoprecipitation with the antibody to CNR/Pedhα (anti-CNR) followed by immunoblotting with anti-Pedhγ-pep, an antibody raised against the Pedhγ-a12 peptide. As shown as in Fig. 2A, Pedhγ was identified as a 100- to 120-kDa band and coimmunoprecipitated with CNR/Pedhα. Similarly, immunoprecipitation of the lysate with anti-Pedhγ-pep followed by immunoblotting with anti-CNRA revealed an association between CNR/Pedhα and Pedhγ. N-cadherin and Pedhγ proteins are reported to be detected in the same fractions of synaptic components and intracellular membranes (15). However, neither CNR/Pedhα nor Pedhγ formed a protein complex with N-cadherin (Fig. 2A). These results indicate that CNR/Pedhα specifically associates with Pedhγ in vivo.

To further confirm the protein interaction between CNR/Pedhα and Pedhγ in vivo, we used two neuroblastoma cell lines, C1300/N1 and NB2a, which endogenously express both proteins. Fig. 2B shows that extracts immunoprecipitated with the anti-CNRA antibody contained CNR/Pedhα and Pedhγ proteins. This result indicated that CNR/Pedhα also specifically associated with Pedhγ in neuroblastoma cells. Both CNR/Pedhα and Pedhγ have putative calcium-binding motifs, which are conserved in the cadherin family. In the classic cadherins, the calcium-binding motifs are necessary for homophilic binding activity. To test the effect of calcium ions on the association between the CNR/Pedhα and Pedhγ proteins, we performed the coimmunoprecipitation analysis in the presence of 1 mM of calcium ions or EDTA. Regardless of the presence of calcium ions or EDTA, CNR/Pedhα associated with Pedhγ, suggesting that the formation of the CNR/Pedhα and Pedhγ complex was independent of calcium ions (Fig. 2C).
The association of CNR/Pcdha and Pedhγ in vivo. A, lysates prepared from mouse brain at postnatal day 7 (P7) were immunoprecipitated with anti-CNRA antibodies, anti-Pedhγ-pep antibodies, or normal rabbit serum (NRS). Immunoprecipitates were resolved on SDS-PAGE, transferred onto PVDF membranes, and probed with anti-CNRA, anti-Pedhγ-pep, or anti-N-cadherin antibodies. B, CNR/Pcdha and Pedhγ were immunoprecipitated from two neuroblastoma cell lines with the anti-CNRA or anti-Pedhγ antibodies. Immunoprecipitation also showed a CNR/Pcdha and Pedhγ association in neuroblastoma cells. C, the association of CNR/Pcdha and Pedhγ was independent of calcium ions. Lysates were prepared from P7 mouse brains in the presence of 1 mM of calcium ions or 1 mM of EDTA and immunoprecipitated with a bacterially produced GST and GST-CNR/Pcdha peptides or anti-N-cadherin antibodies. The purified proteins were then detected by Western blotting with anti-CNRA or anti-Pedhγ antibodies, followed by immunoblotting with anti-CNRA or anti-Pedhγ antibodies. The different forms of CNR/Pcdha and Pedhγ coimmunoprecipitated.

To examine the features of the binding between the CNR/Pcdha and Pedhγ proteins, we used HEK293T cells that were transiently transfected with CNR/Pcdha-v4 (= CNR1 in Ref. 10) or Pedhγ-a12 (= Pedh2c in Ref. 9) or both. Twenty-four hours after transfection, the cells were lysed and their proteins immunoprecipitated using the anti-CNRA or anti-Pedhγ-pep polyclonal antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. The blots were probed with anti-CNRA antibodies. The different forms of CNR/Pcdha and Pedhγ proteins were affinity-purified: the His-tagged and Myc-tagged proteins with the cytoplasmic region deleted, Pcdha-a12delCP and Pedhγ-a12delCP (Fig. 5A). To further examine whether CNR/Pcdha-v4 could be coimmunoprecipitated with other Pedhγs, we used HEK293T cells transfected with CNR/Pcdha-v4 and expression vectors for Pedhγ-a1, -a3, -b2, or -b4. The immunoprecipitation analyses revealed that the CNR/Pcdha-v4 protein could interact with all the Pedhγ proteins tested (Fig. 5B). Similarly, when we examined the binding activity of CNR/Pcdha-v7 protein against each Pedhγ-a1, -a3, -b2, or -b4, the CNR/Pcdha-v7 protein could also associate with all the Pedhγ proteins tested (Fig. 5B). These data suggested that different CNR/Pcdha and Pedhγ proteins can interact with each other.

CNR/Pcdha Associated with Pedhγ at Regions in Their Cytoplasmic and Extracellular Domains—Using PCR amplification from full-length CNR/Pcdha or Pedhγ, we constructed deletion, chimeric, Myc-tagged, or His-tagged constructs and subcloned them into mammalian expression vectors or bacterial expression vectors, as described under “Experimental Procedures.” These protein structures are shown in Fig. 4.

To identify the domain of CNR/Pcdha-v4 responsible for its interaction with Pedhγ, we performed a GST pull-down assay with a bacterially produced GST and GST-CNR/Pcdha-v4CP, which contained only the cytoplasmic domain of CNR/Pcdha-v4, against full-length Pedhγ-a12 or -b2 proteins, or these proteins with the cytoplasmic region deleted, Pedhγ-a12delCP and -b2delCP. We could detect GST-CNR/Pcdha-v4CP binding to the full-length Pedhγ-a12 and -b2 proteins, but not to Pedhγ-a12delCP or -b2delCP (Fig. 5A). The control, GST protein alone, did not interact with any of the Pedhγ proteins used here. These results indicated that the cytoplasmic region of CNR/Pcdha had binding activity with the cytoplasmic region of the Pedhγ proteins.

To further examine the interaction between CNR/Pcdha with Pedhγ, we constructed expression vectors, Pedhγ-EC-His and CNR/Pcdha-EC-Myc, that contained only the extracellular domains of Pedhγ and CNR/Pcdha. The carboxyl terminus of Pedhγ was tagged with Hisα, and that of CNR/Pcdha was tagged with the Myc-epitope. The Pedhγ-a12-EC-His or -b1-EC-His was then transfected into HEK293T cells along with the control vector or CNR/Pcdha-v4-EC-Myc. Forty-eight hours after transfection, the proteins tagged with Hisα or Myc were affinity-purified from the culture medium using a nickel column or Protein G-Sepharose containing immobilized anti-Myc antibody. The purified proteins were then detected by Western blotting with an anti-His or anti-Myc antibody. All the tagged proteins were affinity-purified: the His-tagged and Myc-tagged
proteins were pulled down by the nickel column and anti-Myc antibody, respectively. In addition, the association of CNR/Pcdh/H9251-v4EC-Myc with Pcdh/H9253-a12-EC-His or with -b1-EC-His was also detected in the culture mediums from HEK293 cells coexpressing both CNR/Pcdh/H9251-EC-Myc and Pcdh/H9253-EC-His (Fig. 5B). Thus, the extracellular domains of CNR/Pcdh-EC and Pcdh-EC associated with each other. We also confirmed that the extracellular domain of CNR/Pcdh/H9251-v7 also binds to that of Pcdh-a12 and -b1 (data not shown).

The above results indicated that the extracellular and cytoplasmic regions of the CNR/Pcdhα protein could interact with those of the Pcdhγ protein. To further confirm these interactions, we performed immunoprecipitation assays of the full-length Pcdhα proteins (Pcdhγ-a12 and -b2) with extracellular domain-deleted CNR/Pcdhα-v4EC (Myc-CNR/Pcdhα-v4delEC) or cytoplasmic region-deleted CNR/Pcdhα-v4 (Myc-CNR/Pcdhα-v4delCP). Transfected cells were lysed and immunoprecipitated with an anti-Myc antibody. Both the Pcdhγ-a12 and -b2 full-length proteins were detected in immunoprecipitates from cells transfected with both the extracellular- and cytoplasmic domain-deleted CNR/Pcdhα-v4, using the anti-Myc antibody (Fig. 5C). These data indicated that both the extracellular and

FIG. 4. Deletion mutants and chimeras of CNR/Pcdhα or Pcdhγ used in pull-down assay and transfection experiments. Deletion and chimera constructs of CNR/Pcdhα and Pcdhγ used in the transfection experiments. Myc and His8 are epitope tags used for immunoprecipitation, immunocytochemistry, and Western blotting. For the pull-down assay, the cytoplasmic region of CNR/Pcdhα-v4 was fused with glutathione S-transferase (GST). Sig is the signal sequence; EC is the extracellular domain; TM is the transmembrane domain; and CP is the cytoplasmic domain.

FIG. 5. The extracellular and cytoplasmic domains of Pcdhα and Pcdhγ are responsible for their interaction. A, binding of CNR/Pcdhα-v4 to Pcdhγ-a12 and -b2 through their cytoplasmic regions. Lysates prepared from HEK293T cells expressing full-length protein (upper panel) or cytoplasmic region-deleted mutants (lower panel) of Pcdhγ-a12 or -b1 were incubated with GST (lanes 2 and 5) or GST-CNR/Pcdhα-v4CP (lane 3 and 6). Bound Pcdhγs were detected by immunoblotting. Only Pcdhγs containing the cytoplasmic region were able to bind GST-CNR/Pcdhα-v4CP. Lanes 1 or 4 correspond to the lysates of cells expressing full-length Pcdhγ-a12 or -b1 (upper panel) or cytoplasmic region-deleted mutants (lower panel). Truncated proteins were found in the Pcdhγ-a12 lysates. B, interaction between CNR/Pcdhα and Pcdhγ through their extracellular domains. CNR/Pcdhα-v4EC-Myc, Pcdhγ-a12EC-His, Pcdhγ-b1EC-His, both CNR/Pcdhα-v4EC-Myc and Pcdhγ-a12EC-His, or both CNR/Pcdhα-v4EC-Myc and Pcdhγ-b1EC-His were affinity-purified from the culture medium with a nickel column or Protein G-Sepharose containing immobilized anti-Myc antibody. The purified proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The blots were probed with an anti-His or anti-Myc antibody. C, CNR/Pcdhα-v4 associated with both the extracellular and cytoplasmic domain of Pcdhγ in HEK293T cells. HEK293T cells were transfected with Pcdhγ-a12 or -b2 along with Myc-CNR/ Pcdhα-v4delEC, -delCP, or empty vector. After 24 h, Transfected cells were immunoprecipitated with an anti-Myc antibody followed by immunoblotting with anti-Pcdhγ antibodies.
cytoplasmic domains of CNR/Pcdh could interact with full-length Pcdhγ. There appeared to be slightly more Pcdhγ precipitated with the delCP than with the delEC protein, suggesting that the EC regions were more tightly associated than the cytoplasmic regions.

### Pcdhγ Increased the Cell Surface Expression of CNR/Pcdhα—Previous reports, we demonstrated that CNR/Pcdh-v4 overexpressed in several cell lines was detected mainly in the cytoplasm and hardly on the cell surface (10, 14, 18). Here, we coexpressed CNR/Pcdhα and Pcdhγ in HEK293T cells and observed the colocalization of these proteins. Interestingly, the cellular localization of the Myc-CNR/Pcdhα proteins was different when it was expressed alone versus when it was coexpressed with Pcdhγ-a12 (Fig. 6). Without detergent treatment, the cell surface expression levels of the cells expressing Myc-CNR/Pcdhα-v4 versus those coexpressing Myc-CNR/Pcdhα-v4 with Pcdhγ-a12 were especially different (Fig. 6A, panels c and d). When we used an anti-Myc antibody to detect the Myc-CNR/Pcdhα-v4 protein, the regions of cellular junction were more extensively stained (appearing as lines) in the coexpressing cells (Fig. 6A, panel d) than in the cells expressing Myc-CNR/Pcdhα-v4 alone (Fig. 6A, panel c). The anti-Myc-stained cellular junctions in the coexpressing cells were also stained by the anti-Pcdhγ antibody, suggesting that Myc-CNR/Pcdhα-v4 and Pcdhγ-a12 proteins were colocalized at the cell junctions (double stained in Fig. 6A, panel f). Interactions between these proteins were also detected by immunoprecipitation analysis (Fig. 3). Both CNR/Pcdhα-v4 and Pcdhγ-a12 were concentrated at the intercellular junctions rather than generally on the cell membrane. To further confirm these protein localizations, we used confocal laser-scanning microscopy, which also detected the colocalization of these pro-
Fig. 7. The extracellular domain of Pedhγ promotes the cell surface distribution of CNR/Pedha. A, localization of full-length and deletion mutants of CNR/Pedha-v4 and Pedhγ-a12 proteins in HEK293T cells. HEK293T cells were cotransfected with Myc-CNR/Pedha-v4 (a and d), v4delCP (b and e), or v4delEC (c and f) and empty vector (a-c) or Pedhγ-a12 (d-f). The transfected cells were fixed with 3% paraformaldehyde and labeled with an anti-Myc antibody and with sulfo-NHS-S-S-biotin and extracted with lysis buffer. Biotinylated proteins were then affinity-purified from the lysates with streptavidin-agarose and immunoblotted with the anti-CNR/Pedha or anti-Pedhγ antibody. As shown in A, coexpression with Pedhγ-a12 resulted in an increase in biotinylated CNR/Pedha-v4 and -v7, whereas the amount of biotinylated Pedhγ was little affected by its coexpression with CNR/Pedha. These data indicated that Pedhγ proteins up-regulated the surface expression level of the CNR/Pedha proteins on the plasma membrane. Using Chinese hamster ovary cells, we also found that the extracellular domain of Pedhγ-a12 induced the secretion of the extracellular domain of CNR/Pedha-v4, which was barely transported to the culture medium when expressed alone (data not shown).

Surface Expression Domain of the CNR/Pedha and Pedhγ Proteins—To determine the regions of CNR/Pedha-v4 that inhibited its cell surface expression, we transfected the full-length cDNA or constructs encoding deletion mutants of either the cytoplasmic or extracellular domain of CNR/Pedha-v4 into HEK293T cells and performed immunostaining with an anti-Myc antibody. Immunoreactivity for the full-length protein and the cytoplasmic deletion mutant was detected on the cell surface (Fig. 7A, panels a and b). In contrast, the extracellular domain deletion mutant was not detected on the cell surface (Fig. 7A, panels c and d). This result indicated that the extracellular and transmembrane domains of the CNR/Pedha-v4 protein inhibited its cell surface expression.

The coexpression of Pedhγ enhanced the cell surface expression of the full-length and cytoplasmic deletion mutant of CNR/Pedha-v4. These data indicated that Pedhγ-induced the surface expression of the CNR/Pedha-v4 normally inhibited by the latter’s extracellular and transmembrane domains. Expression of the extracellular domain of CNR/Pedha was sufficient for Pedhγ to enhance its surface expression (data not shown). In contrast, the extracellular domain deletion mutant of CNR/Pedha-v4 showed a down-regulation of surface expression when the Pedhγ protein was coexpressed (Fig. 7A, panels d and f). This result suggested that the cytoplasmic interaction between the CNR/Pedha and Pedhγ proteins (shown in Fig. 5A) inhibits the cell surface expression of CNR/Pedha.

Next, we set out to identify the region of Pedhγ responsible for promoting the cell surface expression of CNR/Pedha. When HEK293T cells were cotransfected with Myc-CNR/Pedha-v4 full-length Pedhγ-a12, or with deletion mutants for its...
cytoplasmic domain (which encoded the extracellular and transmembrane regions), or its extracellular domain, intense immunoreactivity for the Myc-CNR/Pcdh-v4 protein was detected on the cell surface (Fig. 1B). We also detected the induction of the cell surface expression of Myc-CNR/Pcdh-v4 in response to coexpression of the extracellular domain of Pcdh-y-b2 (data not shown). These results demonstrated that interactions between the extracellular domains of the CNR/Pcdh and Pcdh-y proteins are involved in regulating the cell surface expression of CNR/Pcdh.

**DISCUSSION**

Molecules that mediate the specification of different synapses are most likely to be differentially expressed in neuronal populations and to be coupled to intracellular functions at the synaptic plasma membranes. The clustered CNR/Pcdh and Pcdh-y families may meet these criteria (15). These proteins are expressed differentially within single cells (10, 17), mediate cell-cell adhesion (13, 14), and are synaptic proteins that localize in part to synaptic membranes and to the nonsynaptic plasma membrane and the inside of the endosomal vesicles of synapses (10, 15). The CNR/Pcdh and Pcdh-y gene clusters both consist of variable exons and constant exons. The constant exons of both genes encode cytoplasmic tails that are the same for all CNR/Pcdh and Pcdh-y proteins, respectively (10, 11). Here we show that the CNR/Pcdh and Pcdh-y proteins associate with each other through their extracellular and cytoplasmic regions. Their interaction was detected by communoprecipitation using extracts of mouse brain and neuroblastoma. This finding was supported by the partial colocalization of these molecules in cells and their enrichment in the same subcellular fractions. It has been reported that the CNR/Pcdh proteins are barely expressed on the cell surface. However, here we show that Pcdh-y can induce the cell surface expression of CNR/Pcdh. The association of CNR/Pcdh and Pcdh-y in vivo and in vitro was shown for various combinations of these proteins. Because there are 12 and 22 variants in the gene clusters for CNR/Pcdh and Pcdh-y, respectively, there are 264 (12 × 22) possible combinations for protein complexes between them. Their family variants are differentially expressed in neuronal cell populations; therefore, individual neurons could contain different sets of combinational variations. The extracellular and cytoplasmic domains of CNR/Pcdh and Pcdh-y were responsible for their binding, which was independent of calcium ions. It has been reported that, in cotransfected cell lines, N- and R-cadherins form stable cis-heterodimers. The first extracellular cadherin domain of N-cadherin is required for heterodimer formation, and the cis-heterodimer can form in the absence of calcium ions. Furthermore, the cis-heterodimer is a functional unit for mediating cell-cell adhesion (22). Here, we found that the CNR/Pcdh and Pcdh-y family proteins form a protein complex in cotransfected cells. These proteins formed the complexes via their extracellular domains in the absence of calcium and via their cytoplasmic regions. These protein complexes are similar to the cis-heterodimer of R- and N-cadherins with respect to their calcium independence and extracellular domain interaction.

The interaction between CNR/Pcdh and Pcdh-y could be detected in brain extracts; however, the immunostained patterns of CNR/Pcdh and Pcdh-y only partially overlapped and many single-labeled dots were also seen. These data demonstrated that CNR/Pcdh and Pcdh-y did not always interact in vivo. A recent report showed that Pcdh-y is localized to some synapses, to nonsynaptic plasma membranes, and to axonal and dendritic tubulovesicular structures and suggested that these molecules are exchanged among synapses and intracellular compartments (15). It is possible that the double- and single-stained puncta we observed reflect different distributions of CNR/Pcdh and Pcdh-y in synapses and intracellular compartments.

The overlapping staining patterns of the co-overexpressed CNR/Pcdh and Pcdh-y were detected in part in the cell membranes of hippocampal neurons (Fig. 1A). Furthermore, the intercellular junctions were more extensively stained than nonjunctional membranes (Fig. 6A, panel f). Because classic cadherins are concentrated in the cell adhesion sites of intercellular junctions (2), the enrichment of intercellular junctions in these proteins suggested that their hetero-protein complex might have a function in cell adhesion. Some of the Pcdh-y proteins have homophilic cell adhesion activity (23), and the CNR/Pcdh-y proteins have heterophilic cell adhesion activity for β1 integrin, but not homophilic activity (14). HEK293T cells express β1 integrin; therefore, at the intercellular junctions where cell adhesion occurs, a cis-complex of CNR/Pcdh and Pcdh-y at one membrane site could interact with integrins and Pcdh-y in the opposite membrane. Thus, the cis-protein complex of CNR/Pcdh and Pcdh-y might have cell adhesion activity.

The cell surface expression of the CNR/Pcdh protein was repressed by its extracellular domain. This inhibition was removed by the binding of CNR/Pcdh to the extracellular domain of Pcdh-y. In addition, the cytoplasmic region of the CNR/Pcdh protein bound the cytoplasmic region of Pcdh-y. The interactions between these molecules were previously unknown, and may function in mediating intracellular signals. Full-length Pcdh-y induced the surface expression of CNR/Pcdh, resulting in the presence of the CNR/Pcdh and Pcdh-y complex on the cell surface. This protein complex may play a role in the intercellular junctions of the neuronal cell surface.

The molecular mechanisms of the cell surface expression of membrane proteins have been extensively studied. The extracellular, transmembrane, and cytoplasmic regions of membrane proteins have all been implicated in the regulation of their endoplasmic reticulum retention/retrieval, trafficking to their target sites, and their degradation (24). In particular, the retinoid X receptor, KXXX, and di-leucine motifs in the cytoplasmic region of glycoproteins have been shown to regulate the cell surface expression of glycoproteins. Recently, we demonstrated that CNR/Pedho that was overexpressed in a variety of cells was mainly expressed in the endoplasmic reticulum and Golgi (10, 14, 18) and that CNR/Pedho proteins also possess a putative KXXX-like motif in the cytoplasmic region. Here, we showed that extracellular domains of CNR/Pedho proteins negatively regulated their cell surface expression. Therefore, the retention signal of CNR/Pedho is mainly located in its extracellular domain. Only the cytoplasmic region of the CNR/Pedho protein could easily translocate to the plasma membrane and was not retained in the cytoplasm (Fig. 7A); thus, the cytoplasmic domain of the CNR/Pedho proteins (containing the putative KXXX-like motif) did not work as a retention signal of CNR/Pedho.

On HEK293T cells transfected with Pcdh-y, CNR/Pedho was colocalized with Pcdh-y on the plasma membrane. The interaction between the CNR/Pedho and Pcdh-y extracellular domains resulted in an increase in the level of CNR/Pedho cell surface expression. Consistent with these results, it is reported that the cell surface expression of several glycoproteins is promoted by the association of their extracellular domain with the extracellular domain of other glycoproteins and that these complexes form functional receptors. For example, the interaction of calcitonin receptor-like receptor (CLCR) with the receptor activity-modifying proteins (RAMPs) or of Caspr/Paranodin...
with F3 results in the cell surface expression of CLCR or Caspr/Paranodin and induces the formation of functional CLCR/RAMPs or Caspr/F3 receptor (25, 26). The interaction between the extracellular domains of these proteins contributes to their glycosylation and/or trafficking to the cell surface (25, 26). Thus, it is possible that Pedhγ influences the glycosylation and/or trafficking of CNR/Pcdhα to the cell surface via the interaction between the extracellular domains of these proteins.

In the present study, we found a functional interaction between CNR/Pedhα and Pedhγ proteins that causes their cell surface expression. Recently, the deletion of the Pedhγ locus in mice was shown to cause severe apoptosis of the interneurons in the spinal cord and to decrease the number of neuronal cells in the basal forebrain, thalamus, cerebral cortex, and hippocampus (27). The interaction of CNR/Pedhα and Pedhγ that we report here also suggests the possibility that CNR/Pedhα not only contributes to the combinatorial diversity of cell recognition proteins but is also involved in neuronal cell survival and apoptosis in concert with Pedhγ in the brain. However, further analysis will be required to assess the roles of the CNR/Pedhα and Pedhγ protein complex in the development and regulation of the brain.

Acknowledgments—We are grateful to Dr. M. Takeichi (Kyoto University, Japan) for the N-cadherin antibody and H. Masuda for technical assistance.

REFERENCES
1. Yagi, T., and Takeichi, M. (2000) Genes Dev. 14, 1169–1180
2. Takeichi, M. (1991) Science 251, 1451–1455
3. Iwai, Y., Usui, T., Hirano, S., Steward, R., Takeichi, M., and Uemura, T. (1997) Neuron 19, 77–89
4. Tang, L., Hung, C. P., and Schuman, E. M. (1998) Neuron 20, 1165–1175
5. Bozdagi, O., Shan, W., Tanaka, H., Benson, D. L., and Huntley, G. W. (2000) Neuron 28, 245–259
6. Manabe, T., Togashi, H., Uchida, N., Suzuki, S. C., Hayakawa, Y., Yamamoto, M., Yoda, H., Miyakawa, T., Takeichi, M., and Chisaka, O. (2000) Mol. Cell Neurosci. 15, 534–546
7. Lee, C. H., Herman, T., Clandinin, T. R., Lee, R., and Zipursky, S. L. (2001) Neuron 30, 437–450
8. Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O., and Takeichi, M. Neuron 35, 77–89
9. Sano, K., Takihara, H., Heimark, R. L., Ohata, S., Davidson, M., St John, T., Taketani, S., and Suzuki, S. (1993) EMBO J. 12, 2249–2256
10. 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
11. Wu, Q., and Maniatis, T. (1999) Cell 97, 779–790
12. Sugino, H., Hamada, S., Yasuda, R., Tuji, A., Matsuda, Y., Fujita, M., and Yagi, T. (2000) Genomics 63, 75–87
13. Obata, S., Sago, H., Mori, N., Davidson, M., St John, T., and Suzuki, S. T. (1998) Cell Adhes. Commun. 6, 323–333
14. Mutoh, T., Hamada, S., Senzaki, K., Murata, Y., and Yagi, T. (2004) Exp. Cell Res. 294, 494–508
15. Phillips, G. R., Tanaka, H., Frank, M., Elste, A., Fidler, L., Benson, D. L., and Colman, D. R. (2003) J. Neurosci. 23, 5096–5104
16. Tasic, B., Nabholtz, 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
17. Wang, X., Su, H., and Bradley, A. (2002) Genes Dev. 16, 1890–1905
18. Takei, Y., Hamada, S., Senzaki, K., Mutoh, T., Sugino, H., and Yagi, T. (2001) Genomics 72, 321–330
19. Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1980) J. Cell Biol. 86, 831–845
20. Ueda, T., Greengard, P., Berzins, K., Cohen, R. S., Blomberg, F., Grab, D. J., and Siekevitz, P. (1979) J. Cell Biol. 83, 308–319
21. Brewer, G. J., Torricelli, J. R., Evey, E. K., and Price, P. J. (1993) J. Neurosci. Res. 35, 567–576
22. Shan, W. S., Tanaka, H., Phillips, G. R., Arndt, K., Yoshida, M., Colman, D. R., and Shapiro, L. (2000) J. Cell Biol. 148, 579–590
23. Obata, S., Sago, H., Mori, N., Rochelle, J. M., Seldin, M. F., Davidson, M., St John, T., Taketani, S., and Suzuki, S. T. (1995) J. Cell Sci. 108, 3765–3773
24. Ellgaard, L., and Helenius, A. (2003) Rev. Mol. Cell Biol. 4, 181–191
25. McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solar, R., Lee, M. G., and Foord, S. M. (1998) Nature 393, 333–339
26. Fairev-Sarrailh, C., Gauthier, F., Denisenko-Nehrbass, N., Le Bivic, A., Rougon, G., and Girault, J. A. (2000) J. Cell Biol. 149, 491–502
27. Wang, X., Weiner, J. A., Levy, S., Craig, A. M., Bradley, A., and Sanes, J. R. (2002) Neuron 36, 843–854
Interaction with Protocadherin-γ Regulates the Cell Surface Expression of
Protocadherin-α
Yoji Murata, Shun Hamada, Hirofumi Morishita, Tetsuji Mutoh and Takeshi Yagi

J. Biol. Chem. 2004, 279:49508-49516.
doi: 10.1074/jbc.M408771200 originally published online September 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408771200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 26 references, 9 of which can be accessed free at
http://www.jbc.org/content/279/47/49508.full.html#ref-list-1