Regulated ADAM10-dependent Ectodomain Shedding of \(\gamma\)-Protocadherin C3 Modulates Cell-Cell Adhesion*

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\(\gamma\)-Protocadherins (Pcdh\(\gamma\)) are type I transmembrane proteins, which are most notably expressed in the nervous system. They are enriched at synapses and involved in synapse formation, specification, and maintenance. In this study, we show that Pcdh C3 and Pcdh B4 are specifically cleaved within their ectodomains by the disintegrin and metalloprotease ADAM10. Analysis of ADAM10-deficient fibroblasts and embryos, inhibitor studies, as well as RNA interference-mediated down-regulation demonstrated that ADAM10 is not only responsible for the constitutive but also for the regulated shedding of these proteins in fibroblasts and in neuronal cells. In contrast to N-cadherin shedding, which was activated by N-methyl-D-aspartic acid receptor activation in neuronal cells, Pcdh\(\gamma\) shedding was induced by \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate stimulation, suggesting differential regulation mechanisms of cadherin-mediated functions at synapses. Cell aggregation assays in the presence or absence of metalloprotease inhibitors strongly suggest that the ectodomain shedding events modulate the cell adhesion role of Pcdh\(\gamma\). The identifications of ADAM10 as the protease responsible for constitutive and regulated Pcdh\(\gamma\) shedding may therefore provide new insight into the regulation of Pcdh\(\gamma\) functions.

Cadherins are calcium-dependent cell adhesion molecules that play a fundamental role in embryonic and tissue development (1, 2). Protocadherins (Pcdhs) are cadherin-related adhesion molecules with six or seven extracellular cadherin motifs. Their cytoplasmic domains diverge from each other and from those of the classical cadherins, indicating different binding properties and intracellular functions. With 80 members, the Pcdhs represent the largest subgroup of the cadherin superfamily in mammals (3). More than 50 of these genes are arranged in three clusters: Pcdh\(\alpha\), Pcdh\(\beta\), and Pcdh\(\gamma\). The Pcdh\(\beta\) cluster comprises tandemly arrayed single-exon genes flanked by individual promoters, whereas the \(\alpha\) - and \(\gamma\)-clusters additionally contain at the distal 3’ end three small exons coding for a cluster-specific constant domain. The variable domains of the Pcdh\(\alpha\) and Pcdh\(\gamma\) proteins are encoded by large exons and encompass most of the transmembrane protein, including a short cytoplasmic tail. The Pcdhs derived from these gene clusters are predominantly expressed in the nervous system and localized to synaptic junctions (4 – 6). Functionally connected neurons are often characterized by the expression of a distinct set of cadherins, which may thus provide an adhesive code regulating neuronal differentiation and synaptogenesis (7–9). The family-specific constant domains of Pcdh\(\alpha\)- and Pcdh\(\gamma\)-proteins could transduce different extracellular informations into a common intracellular signaling pathway or other executive functions. The large number of distinct Pcdhs possibly contributes to the complexity of neuronal connections by significantly increasing the number of adhesive and/or signaling combinations (7, 10).

Recent reports showed that Pcdh\(\gamma\) proteins undergo proteolytic processing events. A first cleavage is executed by a metalloprotease activity in between the 18 amino acids NH\(\text{2}\)-terminal to the transmembrane segment, releasing a soluble fragment. The precise nature of the Pcdh\(\gamma\) shedding protease is unknown. The proteolytic ectodomain cleavage also leads to a membrane-bound carboxyl-terminal (COOH-terminal) fragment, which is a substrate for regulated intramembrane proteolysis (11, 12). In this process the COOH-terminal membrane-bound fragment becomes a substrate for a presenilin/\(\gamma\)-secretase-mediated proteolysis, resulting in the release of a cytoplasmic COOH-terminal fragment with nuclear localization establishing a role for Pcdh as cell surface signaling molecules. The regulation of regulated intramembrane proteolysis as shown for substrates like Notch, APP, and CD44 is thought to occur at the level of the first ectodomain shedding processing step (13 – 15). This observation and the fact that other regulated intramembrane proteolysis substrates including classical cadherins are cleaved by similar proteases (16, 17) suggested to us that a member of the disintegrin and metalloprotease (ADAM) family is involved in the ectodomain cleavage of Pcdh\(\gamma\).
The ADAMs are a family of type I transmembrane proteins and combine features of both cell adhesion molecules and proteases. They play important roles in fertilization, neurogenesis, and angiogenesis and are involved in the shedding of various membrane-bound proteins including cytokines, growth factors, and adhesion molecules. ADAM10 and ADAM17 (TACE, TNFα-converting enzyme) have been studied in particular in the context of ectodomain shedding. They are involved in the proteolysis of various substrates such as Notch, epidermal growth factor ligands, and fractalkine.

In the present study we focused on the potential role of different ADAMs in Pcdh shedding. We demonstrate that ADAM10 is the prominent protease responsible for Pcdh C3 ectodomain cleavage in fibroblasts, neuronal cells, and mouse embryos. This shedding process modulates Pcdh C3-mediated cell adhesion and is likely to contribute to the suggested intracellular signaling functions of Pcdhγ.

**EXPERIMENTAL PROCEDURES**

*Primary Antibodies and Reagents*—Anti-Pcdhγ C3 antibodies against an extracellular epitope (γC3a, amino acids 71–86) and an intracellular epitope of Pcdh C3 (γC3b, amino acids 792–809) of Pcdhγ C3 were described elsewhere (12). The antibody against the GFP was purchased from Research Diagnostics, Inc. (Concord, MA). ADAM10 was detected using a polyclonal antiserum B42.1 described previously (21). Phorbol 12-myristate 13-acetate (PMA), staurosporine, ionomycin, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate), and NMDA (N-methyl-D-aspartic acid) were purchased from Sigma. MCD (methyl-β-cyclodextrin) was obtained from Research Biochemicals International, TAPI-1, GM6001, and γ-secretase inhibitor L-685,458 from Calbiochem (Bad Soden, Germany). Hydroxamate-based inhibitors GW280264 and GI254023 were described elsewhere (23). CompleteTM EDTA-free protease inhibitor mixture was obtained from Roche Applied Science (Mannheim, Germany).

*Cell Culture and Transfection*—Simian virus large T-antigen-immortalized and primary mouse embryonic fibroblasts (MEFs) cell lines from ADAM9+/–, ADAM10−/−, ADAM15−/–, ADAM17−/– mice, BACE1−/–, and respective wild-type animals were generated and characterized as described elsewhere (21, 22, 24, 25). Constructs encoding Pcdhγ-GFP fusion proteins and stable 293 and K562 transfectants were described previously (5, 12). All cells were grown in DMEM (high glucose) (PAA Laboratories) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The cloning of ADAM10 in pcDNA3.1 vector (Invitrogen, Karlsruhe, Germany) was reported previously (26). Human neuronal H4 cells, kindly provided by J. Wilfang (Erlangen, Germany), and mouse embryonic fibroblasts were transfected in 6-well tissue culture plates with FuGENE 6 (Roche Applied Science) according to manufacturer’s instructions. Primary cultures of hippocampal neurons were prepared in principle as described elsewhere (27). Briefly, the isolated hippocampi were dissociated enzymatically by limited trypsin digestion and mechanically with Pasteur pipettes and plated at a density of 2000–3000 cells/cm² in 6-well tissue culture plates coated with poly-D-lysine (Sigma).

*Pcdh Cleavage Assays*—For analysis of constitutive Pcdh shedding, cells were washed with phosphate-buffered saline and incubated in DMEM with GM6001 (10 μM), TAPI (50 μM), GI254023X (1, 3, 10 μM), GW280264X (1, 3 10 μM), or dimethyl sulfoxide (Me2SO, Roth, Karlsruhe, Germany) for 16 h. For stimulation, fetal calf serum-free medium containing metallo-protease inhibitors or Me2SO was added. The cells were stimulated with PMA (200 ng/ml) for 4 h, with staurosporin (1 μM) for 6 h, with MCD (10 μM) for 60 min, or ionomycin (5 μM) for different time periods. The conditioned media and cells were harvested, and a protease inhibitor mixture (Complete; Roche Applied Science) was added. Supernatants were concentrated (30-fold) through centrifugation in Microcon centrifugal filter devices (YM-10, Millipore, Bedford, MA). Cells were washed with 2 ml of phosphate-buffered saline and harvested in 1 ml of phosphate-buffered saline.

*siRNA Transfection*—The mammalian expression vector pSUPER, kindly provided by Dr. Brummelkamp, was used for expression of siRNA in neuronal H4 cells. The sequence of the human ADAM10 siRNA was as follows: 5′-GACAUUUCAC-CUACGAAU-3′. The sequence was separated by a 9-nucleotide non-complementary spacer (tctcttgaa) from the corresponding reverse complement of the same 19-nucleotide sequence. These sequences were inserted into the pSUPER backbone after digestion with BglII and HindIII. H4 cells were transfected with pSuper-ADAM10-siRNA vector with the use of FuGENE 6 (Roche Applied Science) according to manufacturer’s recommendations.

*SDS-PAGE and Western Blotting*—Cells were lysed in Triton lysis buffer (1% Triton X-100, 5 mM Tris, 1 mM EGTA, 250 mM saccharose, pH 7.4). Protein concentrations were determined with a BCA protein assay (Pierce). Equal amounts of protein were loaded on 10% SDS gels for analysis of Pcdh C3 stubs. The samples were electrotransferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Freiburg, Germany), which were blocked overnight with 5% skim milk in Tris-buffered saline (TBS). After incubation with the indicated antibody in blocking buffer, membranes were washed three times in TBS containing 0.1% Tween (TBS-T). Primary antibodies were detected using affinity-purified peroxidase-conjugated secondary antibodies for 1 h at room temperature. Detection was carried out using the ECL detection system (Amersham Biosciences). Signals were recorded by a luminescent image analyzer (Image Reader LAS1000, Fujifilm, Tokyo, Japan) and analyzed with the image analyzer software (Gel-ProAnalyser, Media Cybernetics, Silver Spring, MD). For probing blots, polyvinylidene difluoride membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 1 h at 60 °C with occasional shaking. After three washes in large volumes of TBS-T, membranes were blocked in blocking buffer for 1 h at room temperature, and immunodetection was repeated.

*Cell Aggregation Assays*—For cell aggregation assays either wild-type K562 cells or K562 cells stably expressing Pcdhγ C3 were used as described previously (5). Briefly, single cell suspensions were produced by two passages through yellow
FIGURE 1. γ-Protocadherin C3 shedding is sensitive to metalloprotease inhibition. A, schematic representation of Pcdhγ C3 cleavage sites and antibody binding regions. Full-length 120-kDa Pcdhγ C3 is cleaved by metalloprotease activity in a NH2-terminal 100-kDa (NTF) and a COOH-terminal membrane-bound 25-kDa fragment (CTF1), which can be further processed by γ-secretase like activity in soluble 20-kDa fragments (CTF2). B, wild-type fibroblasts were treated with dimethyl sulfoxide (DMSO), broad-spectrum metalloprotease inhibitors TAPI, or GM6001 (left panel), ADAM10 inhibitor (GI254023X) (middle panel), and ADAM10/ADAM17 inhibitor (GW280264X) (right panel), respectively. The generation of the 25-kDa Pcdhγ C3-CTF1 was inhibited after treatment of the cells with broad-spectrum metalloprotease inhibitors TAPI or GM6001 and after GI254023X treatment. C, the effect of the different inhibitors on the generation of the 25-kDa Pcdhγ C3-CTF1 was quantified by densitometric analysis of full-length (FL) Pcdhγ C3 (B) and COOH-terminal fragment of Pcdhγ C3 (CTF1). Data represent mean ± S.E. of three independent experiments. *, p < 0.05 versus dimethyl sulfoxide (DMSO).
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**FIGURE 2. Involvement of ADAM10 in constitutive shedding of γ-protocadherins.** A, constitutive Pcdhγ C3 cleavage is reduced in ADAM10 Ang/ mice. Representative immunoblot with total cell extracts from ADAM9 Ang/, ADAM10 Ang/, ADAM15 Ang/, ADAM17 Ang/, and BACE1 Ang/ mouse embryonic fibroblasts (mouse embryonic fibroblasts). Supernatants of these cells were also subjected to Western blot analysis using NH2-terminal anti-Pcdh antibody (C3a) antibodies (lower panel). FL, full-length Pcdhγ C3; CTF1, COOH-terminal fragment 1. Right panel, CTF generation was calculated as percentage of total Pcdhγ C3 (full-length Pcdhγ C3 plus CTF1) by densitometric analysis. Results were obtained from six independent experiments and expressed as mean ± S.E. The Pcdhγ C3-CTF1 generation was significantly decreased only in ADAM10 Ang/ MEFs as compared with wild-type cells (*p < 0.05 versus wild-type cells). B, anti-Pcdhγ C3 (COOH-terminal, C3b) immunoblot of proteins extracted from three independent ADAM10 Ang/ and wild-type MEFs. C, ADAM10-deficient cells were transfected with wild-type mouse ADAM10 (A10+/+) and compared with wild-type and ADAM10-deficient MEFs for ADAM10 and Pcdhγ expression. The immunoblot shows one representative out of three independent experiments. FL, full-length Pcdhγ C3; CTF1, COOH-terminal fragment of Pcdhγ C3; FL, wild-type MEFs; C, ADAM10-deficient mice were transfected with constructs encoding Pcdhγ C3 fused to GFP at either the COOH terminus (left panel) or the NH2 terminus (right panel). D, full-length or cleaved Pcdhγ C proteins were visualized by anti-GFP antibodies. E, ADAM10-deficient cells were transfected with COOH-terminal tagged Pcdhγ B4-GFP and compared with Pcdhγ B4-GFP transfected wild-type MEFs for Pcdhγ B4-GFP expression.

The full-length 120-kDa Pcdhγ C3 is cleaved in the extracellular domain by a protease activity, generating a 25-kDa COOH-terminal fragment termed CTF1, which can be further processed by a γ-secretase-like activity into a CTF2 of 20 kDa (Ref. 12 and Fig. 1A). Pcdhγ C3 is endogenously expressed in MEFs. Under steady state conditions, the majority of Pcdhγ C3 was proteolytically cleaved in MEFs as indicated by the ratio between Pcdhγ C3-CTF1 and total Pcdhγ C3 (Fig. 1B, left panel, and Fig. 1C). To identify the protease responsible for the first rate-limiting processing step in the regulated intramembrane proteolysis of Pcdhγ C3 we compared metalloprotease inhibitors with different inhibitory profiles for their ability to inhibit Pcdhγ C3 processing in MEFs. Inhibitors such as GM6001 and TAPI, pipette tips (10 μl, Sarstedt). Viability of cells was determined by trypan blue dye exclusion and shown to be >95% in every assay. One million cells of each transfectant clone or wild-type cells were incubated in 3 ml of DMEM medium containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C. For stimulation, metalloprotease inhibitor GM6001 or Me2SO were added. After 16 h, the cells were incubated with PMA (200 ng/ml) for a further 4 h. Afterward, cells were photographed using an inverted phase-contrast microscope (Carl Zeiss) and counted manually for both single cells and number of cells in aggregates. Average standard deviations of one representative experiment were calculated.

**RESULTS**

**Inhibitor Studies Suggest an Involvement of ADAM10 in γ-Protocadherin C3 Shedding**—The full-length 120-kDa Pcdhγ C3 is cleaved in the extracellular domain by a protease activity, generating a 25-kDa COOH-terminal fragment termed CTF1, which can be further processed by a γ-secretase-like activity into a CTF2 of ~20 kDa (Ref. 12 and Fig. 1A). Pcdhγ C3 is endogenously expressed in MEFs. Under steady state conditions, the majority of Pcdhγ C3 was proteolytically cleaved in MEFs as indicated by the ratio between Pcdhγ C3-CTF1 and total Pcdhγ C3 (Fig. 1B, left panel, and Fig. 1C). To identify the protease responsible for the first rate-limiting processing step in the regulated intramembrane proteolysis of Pcdhγ C3 we compared metalloprotease inhibitors with different inhibitory profiles for their ability to inhibit Pcdhγ C3 processing in MEFs. Inhibitors such as GM6001 and TAPI,
both known to inhibit a wide spectrum of metalloproteases, led to a significant reduction of the 25-kDa CTF (Fig. 1, B and C, left panel).

Previously, we have described two hydroxamate-based compounds that differ in their capacity to block the activities of the two disintegrin-like metalloproteases, ADAM17 and ADAM10 (28). The inhibitor GW280264X has been shown to preferentially block ADAM17 and to a lesser extent ADAM10, while the compound GI254023X preferentially blocked ADAM10. Western blot analysis of cell lysates of inhibitor-treated wild-type MEFs demonstrated that the ADAM10 inhibitor GI254023X caused a reduction in Pcdh C3-CTF1 levels in wild-type but not in ADAM10-deficient fibroblasts. Cells were stimulated with 5 μM ionomycin for different periods in the presence or absence of GI254023X (5 μM). Cell pellets were harvested and analyzed by Western blotting using COOH-terminal anti-Pcdh C3 antibodies. FL, full-length Pcdh C3; CTF1, COOH-terminal fragment of Pcdh C3.

To further analyze whether ADAM10 mediates shedding of Pcdh family members in general, we used wild-type fibroblasts and ADAM10-deficient cells expressing different Pcdh proteins fused COOH-terminally to GFP. Whereas cleavage of the Pcdh C3-GFP protein was observed in wild-type fibroblasts, a clear reduction in the generation of the Pcdh C3-GFP-CTF1 was noted in ADAM10-deficient cells (Fig. 2D) confirming our findings on the endogenous Pcdh. Analysis of Pcdh B4-GFP, another Pcdh family member, showed the same pattern: a strikingly reduced generation of COOH-terminal fragments in ADAM10-deficient cells compared with wild-type fibroblasts (Fig. 2E). This provides evidence for a general role of ADAM10 in mediating the extracellular cleavage of the different members of the Pcdh family.

ADAM10 Is Also Crucial for Induced Shedding of γ-Protocadherin—In general, shedding of surface membrane proteins can occur in a constitutive and in a regulated fashion (16, 26, 30). Therefore we set out to analyze in more detail the regulated shedding of Pcdh C3 (Fig. 3A). Stimulation of protein kinase C using the phorbol ester PMA (31) strongly induced Pcdh C3 shedding in wild-type fibroblasts. Staurosporine, which

FIGURE 3. ADAM10 involvement in stimulated Pcdh γ C3 shedding. A, effect of different stimuli on Pcdh γ C3 shedding. Cells were stimulated with PMA (100 ng/ml), methyl-β-cyclodextrin (MCD, 10 mM) or vehicle control (dimethyl sulfoxide (DMSO)) for 4 h, with ionomycin (IM, 5 μM) for 30 min or with staurosporine (SP, 1 μM) for 6 h in the presence of γ-secretase inhibitor (0.5 μM). Subsequently, cell pellets were lysed and subjected to Pcdh γ C3 (COOH-terminal) Western blot analysis. Data represent mean ± S.E. of three independent experiments. *, p < 0.05 versus dimethyl sulfoxide (DMSO)-treated wild-type cells; †, p < 0.05 versus wild-type cells. B, ionomycin-induced Pcdh γ C3 shedding occurred in wild-type but not in ADAM10-deficient fibroblasts. Cells were stimulated with 5 μM ionomycin or vehicle control (dimethyl sulfoxide (DMSO)) for different periods in the presence or absence of GI254023X (5 μM). Cell pellets were harvested and analyzed by Western blotting using COOH-terminal anti-Pcdh γ C3 antibodies. FL, full-length Pcdh γ C3; CTF1, COOH-terminal fragment of Pcdh γ C3.
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Subsequently, cells were lysed and analyzed by Western blotting. The blot was probed with anti-ADAM10 (17) and N-cadherin (16), also stimulated Pcdh activation of metalloprotease-mediated cleavage of E-cadherin induces apoptosis and has been previously implicated in the activation of metalloprotease-mediated cleavage of E-cadherin (17) and N-cadherin (16), also stimulated Pcdhγ C3 shedding. Also ionomycin, an agent that promotes shedding of cadherins through stimulation of calcium influx (16), increased CTF1 production in wild-type cells. Depletion of cholesterol using cyclodextrin (MCD) stimulated the shedding of Pcdhγ C3 only slightly (Fig. 3A, left panel).

This clearly shows that Pcdhγ C3 cleavage is controlled by several signaling pathways, which include protein kinase C- and calcium-dependent regulation. We next addressed whether this inducible cleavage is dependent on ADAM10 by analyzing the effects of these different compounds in ADAM10-deficient cells (Fig. 3A, middle panel). If another protease (e.g. TACE) was responsible for the induction of Pcdhγ shedding, we expected to observe CTF1 generation in these cells, which was not the case as shown by analysis of three independent experiments (Fig. 3A). We therefore conclude that ADAM10 is also responsible for the stimulated shedding of Pcdhγ C3.

It has been demonstrated that calcium influx is temporally coincident with synapse formation and leads to a redistribution of synaptic proteins during synapse maturation (32, 33). Extracellular influx of calcium also induces rapid cleavage of classical cadherins (e.g. E-cadherin and N-cadherin) (16, 17). Therefore we investigated this aspect in more detail. In wild-type cells, ionomycin induced an accumulation of the CTF1 within minutes (Fig. 3B, left panel). This effect was completely blocked with the ADAM10 inhibitor GI254023X in wild-type cells (Fig. 3B, middle panel) and also absent in ADAM10-deficient cells (Fig. 3B, right panel). Taken together, these results demonstrate that ADAM10 is the major protease responsible for the constitutive and for the inducible Pcdhγ C3 shedding.

ADAM10-dependent γ-Protocadherin Cleavage in Neuronal Cells—Pcdhγ C3 as well as ADAM10 are predominately expressed in neuronal cells. Pcdhγ C3 possibly participates in neuronal differentiation and regulation of synaptogenesis (34, 35). Like MEFs, human neuroglioma H4 cells express Pcdhγ C3 and generate a 25-kDa CTF1 proteolytic fragment recognized by our C3-specific antibodies (Fig. 4A).

Upon ADAM10 overexpression in these cells (Fig. 4A, upper panel) an increased level of Pcdhγ C3-CTF1 was observed (Fig. 4A, lower panel). We then investigated whether this inducible cleavage is dependent on ADAM10 by analyzing the effects of these different compounds in ADAM10-deficient cells (Fig. 4B, middle panel). If another protease (e.g. TACE) was responsible for the induction of Pcdhγ shedding, we expected to observe CTF1 generation in these cells, which was not the case as shown by analysis of three independent experiments (Fig. 4B, lower panel). We therefore conclude that ADAM10 is also responsible for the stimulated shedding of Pcdhγ C3.

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4A, lower panel). We confirmed the role of endogenously expressed ADAM10 in Pcdhγ C3 shedding in neuronal cells using vector based RNA interference (36). ADAM10 levels dropped to 15%, and Pcdhγ C3-CTF1 levels dropped to 35%, as compared with the levels seen in mock transfected cells (Fig. 4B). To clarify whether PMA stimulates ADAM10-mediated shedding also in H4 cells, we analyzed the effect of PMA treatment on Pcdhγ C3 cleavage in the presence or absence of the specific inhibitors GI254023X and GW280264X in these cells. PMA stimulation increased the amount of Pcdhγ C3-CTF1 generation (Fig. 4C). By contrast, no increase in the amount of Pcdhγ C3-CTF was seen when the ADAM10 inhibitor GI254023X was added to the cells prior to PMA stimulation. The treatment with the ADAM10/ADAM17 inhibitor GW280264X showed only a minor effect. These results were confirmed by statistical analysis of three different experiments (Fig. 4C, right panel) suggesting that ADAM10 is also involved in the PMA-stimulated cleavage of Pcdhγ C3 in neuronal cells.

ADAM10-mediated γ-Protocadherin C3 Shedding Cannot Be Compensated in Vivo—To further elucidate the in vivo relevance of Pcdhγ C3 cleavage by ADAM10 we analyzed extracts of E9.5 wild-type and ADAM10-deficient embryos by Western blotting. It has been shown recently that the processing of Pcdhγ involves a presenilin-dependent downstream processing of Pcdhγ C3-CTF1, resulting in a second COOH-terminal cleavage product, termed Pcdhγ C3-CTF2 (12). In addition to the Pcdhγ C3 full-length protein, we found both fragments, CTF1 and a smaller fragment, most likely corresponding to CTF2 in the wild-type embryos. The generation of both Pcdhγ C3 fragments was almost, if not completely, abolished in the ADAM10-deficient embryos even though the full-length protein was expressed in equal amounts (Fig. 5). These findings indicate that the ADAM10-dependent formation of Pcdhγ C3-CTF1 is a prerequisite for further processing by the γ-secretase complex. Thus, these results show that ADAM10 is essentially involved in Pcdhγ C3 processing and signaling in vivo.

Glutamate Stimulation Induces ADAM10-mediated γ-Protopcadherin C3 and N-cadherin Shedding in Neuronal Cells—Dynamic regulation of synaptic efficacy is thought to play a critical role in the formation of neuronal circuitry during development and learning (37). To evaluate whether stimulation of neural activity is able to induce ADAM10-mediated processing of Pcdhγ C3, we incubated H4 cells with KCl and glutamate to mimic depolarization and glutamate receptor stimulation. Application of both stimuli in H4 cells resulted in a second COOH-terminal cleavage product, termed Pcdhγ C3-CTF2 (12). In addition to the Pcdhγ C3 full-length protein, we found both fragments, CTF1 and a smaller fragment, most likely corresponding to CTF2 in the wild-type embryos. The generation of both Pcdhγ C3 fragments was almost, if not completely, abolished in the ADAM10-deficient embryos even though the full-length protein was expressed in equal amounts (Fig. 5). These findings indicate that the ADAM10-dependent formation of Pcdhγ C3-CTF1 is a prerequisite for further processing by the γ-secretase complex. Thus, these results show that ADAM10 is essentially involved in Pcdhγ C3 processing and signaling in vivo.

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To more precisely define the pathway of glutamate-dependent induction of Pcdhγ C3 shedding in H4 cells and primary neurons, we compared the effect of glutamate in the presence or absence of the selective AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid)/kainate receptor antagonist CNQX, which blocks non-NMDA-type glutamate receptors. Interestingly, the increase of Pcdhγ C3 proteolysis after stimulation with glutamate or KCl was strongly diminished in the presence of CNQX (Fig. 6B, left panel). Thus, we conclude that the glutamate-induced response is mediated by non-NMDA/AMPA-type receptors. These findings were confirmed in primary neurons, where the application of AMPA, but not of NMDA, led to an increased processing of Pcdhγ C3 (Fig. 6B, right panel).

γ-Protopcadherin C3-mediated Cell-Cell Aggregation Is Regulated by Metalloprotease Cleavage—Cadherins have been shown to function as intercellular adhesion molecules (1, 2, 38–40). Recently, we showed that Pcdhγ C3, when stably expressed in cells, is able to promote cell-cell aggregation (5). Wild-type lymphoid K562 cells show only minor cell-cell aggregation in culture (Ref. 41 and Fig. 7A), whereas increased numbers of cell aggregates are found in K562 cells stably expressing Pcdhγ C3 (Ref. 5 and Fig. 7E).

To further elucidate the functional relevance of metalloprotease-mediated Pcdhγ C3 shedding for cell-cell aggregation, we used GM6001 as a broad-spectrum metalloprotease inhibitor and the shedding-inducing agent PMA. Cell aggregation of stably transfected K562 cells was strongly increased by the inhibitor GM6001 (Fig. 7F), whereas GM6001 treatment showed only a slight effect on K562 cell aggregation in untransfected cells (Fig. 7B). To examine whether increased metalloprotease-mediated shedding, evoked by PMA stimulation, leads to an anti-adhesive effect, we analyzed cell aggregation after a 4-h presence of PMA. PMA decreased cell adhesion by 20% compared with Me2SO-treated Pcdhγ C3 cells, while minor changes were seen in wild-type K562 cells (Fig. 7C and G). Pretreatment with GM6001 prior to PMA stimulation abolished the PMA-induced effect in

**FIGURE 5. Loss of Pcdhγ C3 ectodomain shedding in ADAM10-deficient embryos.** Same amounts of protein from total wild-type embryo and ADAM10−/− embryo extracts (E9.5) were analyzed by immunoblot for ADAM10 expression and for Pcdhγ C3 cleavage products. FL, full-length Pcdhγ C3; CTF1, COOH-terminal fragment 1 of Pcdhγ C3; CTF2, COOH-terminal fragment 2 of Pcdhγ C3; p, precursor of ADAM10; m, mature form of ADAM10. A representative experiment out of two experiments is shown.

**FIGURE 6. Glutamate stimulation induces Pcdhγ C3 shedding in H4 cells.** A, immunoblot of Pcdhγ C3 shedding in the presence or absence of the selective AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid)/kainate receptor antagonist CNQX, which blocks non-NMDA-type glutamate receptors. Interestingly, the increase of Pcdhγ C3 proteolysis after stimulation with glutamate or KCl was strongly diminished in the presence of CNQX (Fig. 6B, left panel). Thus, we conclude that the glutamate-induced response is mediated by non-NMDA/AMPA-type receptors. These findings were confirmed in primary neurons, where the application of AMPA, but not of NMDA, led to an increased processing of Pcdhγ C3 (Fig. 6B, right panel).
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FIGURE 6. Glutamate stimulation induces ADAM10-mediated Pcdhγ C3 and N-cadherin shedding in neuronal cells. A, H4 cells were incubated for 30 min in the presence or absence of different neuronal receptor agonists (NMDA, 50 μM; glutamate, 50 μM; KCl, 5 mM) and analyzed for Pcdhγ C3 (left) or anti-N-cadherin shedding (right). FL and CTF1 indicate the positions of full-length and carboxyl-terminal fragments, respectively. Data represent mean ± S.E. of three independent experiments. *, p = 0.05 significant difference from dimethyl sulfoxide (DMSO). B, H4 cells or primary neurons were incubated with different neuronal receptor agonists in the presence or absence of selective AMPA/kainate receptor antagonist CNQX (10 μM). Cellular extracts were analyzed by Western blot using anti-Pcdhγ C3 antibodies. Data represent mean ± S.E. of three independent experiments. *, p < 0.05 significant difference from dimethyl sulfoxide (DMSO). ADAM10 activity has been implicated in the constitutive shedding of several substrates. However, shedding induced upon stimulation may be attributed to different members of the ADAM family, e.g. to ADAM17 (22). For Pcdhγ, we demonstrate that both constitutive and inducible shedding are dependent on the presence of ADAM10. For example, calcium influx triggered by the application of ionomycin led to a strong stimulation of ADAM10-mediated Pcdhγ C3 shedding. This finding is of particular interest in the context of synapse formation since modulation of intracellular calcium levels is important for synapse shaping and regulation of synaptic efficacy (42, 43). Induced shedding of cadherin and protocadherin ectodomains may provide an elegant mechanism to combine the local control of synaptic adhesion with intracellular signaling as a result of downstream processing of the membrane-bound CTFs via the γ-secretase complex. Regulated processing of E- and N-cadherin leads to a cascade of distinct signaling events in the cytosol (16, 17). In contrast, processing of Pcdhγ C3 by the γ-secretase generates a CTF2, which is analogous to the situation described for Notch-ICD and localizes to the nucleus (11, 12). Notch controls transcriptional activation or repression of its direct target genes by binding to downstream transcription factors (44). Our analysis of

Pcdhγ C3 K562 cells (Fig. 7, D and H). Taken together, these findings suggest that metalloprotease-mediated shedding of Pcdhγ C3 is involved in regulating Pcdhγ C3-dependent cell-cell aggregation.

DISCUSSION

Pcdhγ are widely expressed during the development of the central nervous system. Roles in cell-cell adhesion and signaling have been attributed to these cadherin-like molecules (3, 34). Important contributions of Pcdhγ proteins to the proper development of the central nervous system are apparent by the strong neurological phenotype and neonatal death of mice lacking a functional Pcdhγ cluster (11), but details of the involved functional pathways are largely unknown.

Pcdhγ are endoproteolytically cleaved by a metalloprotease to generate a membrane-bound CTF and a soluble NTF, which is released to the extracellular environment. The metalloprotease cleavage is a prerequisite for subsequent intramembrane presenilin-mediated processing and release of a smaller CTF with nuclear localization and potential signaling functions (11, 12). In this report we show that the metalloprotease ADAM10 is critically involved in the ectodomain shedding of Pcdhγ proteins. We find that constitutive shedding of Pcdhγ C3 is strongly reduced in ADAM10-deficient fibroblasts, whereas the shedding is unchanged in fibroblast deficient for other members of the ADAM family. Accordingly, the ADAM10 inhibitor compound GI2540223X is able to block constitutive shedding of Pcdhγ C3, yielding similar or even stronger effects than the broad-spectrum metalloprotease inhibitors TAPI and GM6001, which we and others have used previously (11, 12). In line with these findings ectodomain cleavage of Pcdhγ C3 is also absent in ADAM10 knockout embryos. The Pcdhγ cluster encodes 22 different family members, and a first analysis of several family members indicated that Pcdhγ in general is a substrate for regulated proteolytic processing. This would allow the propagation of different types of intracellular signals initiated by ectodomain shedding (12).

ADAM10 activity has been implicated in the constitutive shedding of several substrates. However, shedding induced upon stimulation may be attributed to different members of the ADAM family, e.g. to ADAM17 (22). For Pcdhγ, we demonstrate that both constitutive and inducible shedding are dependent on the presence of ADAM10.
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FIGURE 7. Aggregation in stably Pcdhγ C3-transfected K562 cells is increased after treatment with metalloprotease inhibitor GM6001. Aggregation capacity of Wt-K562 suspension cells (A–D) and K562 cells stably expressing Pcdhγ C3-GFP (E–H), While Wt-K562 suspension cells showed only slight aggregation in cell culture (A), increased aggregation of K562 cells stably expressing Pcdhγ C3-GFP was observed (E). Incubation with GM6001 (10 µM) for 16 h only slightly affected aggregate formation in WT-K562 cells (B) but led to significantly increased cell aggregation of K562-Pcdhγ C3-GFP cells (F) compared with the dimethyl sulfoxide (DMSO) control, respectively. PMA stimulation (200 ng/ml) for 4 h decreased cell aggregation of WT-K562 cells (C) and affected the aggregation capacity of K562-Pcdhγ C3-GFP cells (G). When WT-K562 cells were incubated in the presence of GM6001 (10 µM) for 16 h and stimulated with PMA (200 ng/ml) for an additional 4 h, the PMA effect was abrogated (D and H). I, quantification of aggregated cells as compared with total number of cells. Cells were counted manually for both single cells and number of cells in aggregates (consisting of more than four cells). A representative experiment out of three experiments is shown. Values are expressed as standard deviations.

ADAM10-deficient embryo extracts demonstrate that the ADAM10-dependent cleavage of Pcdhγ is a prerequisite, thus a rate-limiting step for intramembrane proteolysis of Pcdhγ-C3 also in vivo and suggests an important function of ADAM10 in the regulation of Pcdhγ-mediated signaling. Further analysis of neuronal cell lines and primary neurons supported this view since we found a specific activation of Pcdhγ-C3 shedding upon activation of non-NMDA-type glutamate receptors by the agonist AMPA. In contrast, activation of NMDA-type glutamate receptors had no effect on Pcdhγ C3 shedding but stimulated N-cadherin shedding as we have also reported previously (16). These findings indicate that regulation of the interactions of ADAM10 with its substrates, e.g. Pcdhγ C3 or N-cadherin, is able to specifically determine downstream signaling.

As a result of ADAM10-mediated cleavage accumulation of soluble Pcdhγ-NTF in the culture medium is observed (Ref. 12 and Fig. 2A). Regulated processing and nuclear translocation of Pcdhγ-NTF promoted the idea that Pcdhγ proteins may function as cell surface receptors; however, bi-directional signaling mechanisms, which involve Pcdhγ-NTF, could be envisioned as well. In particular, Pcdhγ-NTFs may engage in heterophilic binding to neuronal receptor molecules, thus acting as ligands in a paracrine or autocrine fashion, similar to the situation shown for other ADAM10 substrates, e.g. the various ligands of the epidermal growth factor receptor (22). In addition, modulation of cell motility or cell adhesion may result from (homophilic) binding of soluble Pcdhγ-NTF to membrane-tethered Pcdhγ complexes, as we demonstrated previously for E-cadherin-NTF generated by ADAM10 activity (17).

Classical cadherins were initially identified as the molecules that mediate cell-cell adhesion, and knock-out mice experiments have proven the role of classical cadherins in embryogenesis (45). They provide strong and usually homophilic cell-cell adhesion, which is important for tissue formation during embryogenesis or cell differentiation, e.g. in the nervous system (8). Similarly, protocadherins, including the Pcdhγ family, were shown to mediate cell-cell adhesion, which is, however, much weaker than that of classical cadherins (3, 5). Metalloprotease-mediated ectodomain shedding may limit the amount of adhesive moi-
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diated signaling and cell adhesion. This opens novel perspectives to study Pcdhγ functions in tissue formation, neuronal differentiation, and synaptogenesis.

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