Ectodomain Shedding of the Neural Recognition Molecule CHL1 by the Metalloprotease-disintegrin ADAM8 Promotes Neurite Outgrowth and Suppresses Neuronal Cell Death*

Silvia Naus‡‡, Melanie Richter‡‡, Dirk Wildeboer‡, Marcia Moss§§*, Melitta Schachner*†, and Jörg W. Bartsch‡‡‡

From the §Entwicklungbiologie & Molekulare Pathologie, W7, Universität Bielefeld, 33501 Bielefeld, Germany, the ¶Zentrum für Molekulare Neurobiologie, Universität Hamburg, Martinistrasse 52, 20246 Hamburg, Germany, and **Biozyme Inc., Apex, North Carolina 27523

The neural cell adhesion molecule “close homologue of L1,” termed CHL1, has functional importance in the nervous system. CHL1 is expressed as a transmembrane protein of 185 kDa, and ectodomain shedding releases soluble fragments relevant for its physiological function. Here we describe that ADAM8, a member of the family of metalloprotease-disintegrins cleaves a CHL1-Fc fusion protein in vitro at two sites corresponding to release of the extracellular domain of CHL1 in fibronectin (FN) domains II (125 kDa) and V (165 kDa), inhibited by batimastat (BB-94). Cleavage of CHL1-Fc in the 125-kDa fragment was not detectable under non-reducing conditions arguing that cleavage resulting in the 165-kDa fragment is more relevant in releasing soluble CHL1 in vivo. In cells transfected with full-length ADAM8, membrane proximal cleavage of CHL1 was similar and not stimulated by phorbol ester 12-O-tetradecanoylphorbol-13-acetate and pervanadate. No cleavage of CHL1 was observed in cells expressing either inactive ADAMs with a Glu330 to Gln exchange (EQ-A8), or active ADAM10 and ADAM17. Consequently, processing of CHL1 was hardly detectable in brain extracts of ADAM8-deficient mice but enhanced in a neurodegenerative mouse mutant. CHL1 processed by ADAM8 in supernatants of COS-7 cells and in co-culture with cerebellar granule neurons was very potent in stimulating neurite outgrowth and suppressing neuronal cell death, not observed in cells co-transfected with CHL1/EQ-A8, CHL1/ADAM10, or CHL1/ADAM17. Taken together, we propose that ADAM8 plays an important role in physiological and pathological cell interactions by a specific release of functional CHL1 from the cell surface.

Many membrane-anchored proteins are subjected to proteolytic processing thereby releasing their extracellular domains, a process termed ectodomain shedding (1). This modification causes qualitative and irreversible changes in the function of these molecules. As demonstrated with genetic and biochemical means, enzymes capable of these functions are ADAMs (2) (for “a disintegrin and metalloprotease domain”), proteins that constitute a family of transmembrane glycoproteins with essential physiological roles in fertilization, myogenesis, and neurogenesis (2). These functions are due to distinct protein domains involved in either cell-cell fusion or cell-cell interaction, or to their zinc-coordinating metalloprotease domain. To date, the family of ADAM proteinases comprises more than 30 members in different species (3, 4). Fourteen of the murine ADAMs contain the catalytic ensemble sequence HEXXHXXXXHDX in their metalloprotease domains and are therefore predictably proteolytically active (3, 4). The cleavage of myelin basic protein (MBP) by ADAM10/MADM (mammalian disintegrin-metalloprotease) was the first demonstration of proteolysis by ADAMs (5). The TNF-α convertase (TACE/ADAM17) was purified on the basis of its ability to cleave TNF-α (6, 7) and a number of other peptide and protein substrates in vitro (1, 8). TACE is also implicated in the shedding of factors in the EGF ligand family, such as heparin-binding epidermal growth factor (HB-EGF) and amphiregulin (reviewed in Ref. 2), and the physiological importance of this cleavage was demonstrated by similar defects in TACE and HB-EGF-deficient mice (9, 10).

In the CNS, proteolytic processing of the amyloid precursor protein (APP (11)) is an essential event in the pathology of Alzheimer’s disease. As α-secretases, a number of ADAMs have been described to cleave APP at the respective α-cleavage site, ADAM9, ADAM10, and ADAM17, suggesting that ADAM family members serve essential roles in the CNS. Recently, ADAM8, an ADAM originally cloned from monocytic cells (12), has been shown to be expressed in the CNS in neurons, reactive glia cells (astrocytes and microglia), and oligodendrocytes (13) and was described as a sheddase of the low affinity IgE receptor CD23 (14). In addition to substrates of ADAM8 in the immune response, a specific substrate of ADAM8 in the CNS has not yet been described. From these examples it is clear, that for each substrate and tissue, the physiological relevance of processing has to be proven by a
Ectodomain Shedding of CHL1

series of biochemical methods. In the CNS, neural recognition molecules play important roles in specifying cell interactions during development, regeneration, and modification of synaptic activity. One subfamily of recognition molecules is the immunoglobulin (Ig) superfamily. In their extracellular parts these molecules contain IgG-like domains with essential cysteine residues and four to five fibronectin repeats (FN). Within these FN domains, cell adhesion molecules can be proteolytically processed and released from the cell membrane, thereby exerting important biological functions. The first identified member of the Ig family was L1 (15). L1 is a 200- to 220-kDa type I transmembrane protein. In the mouse brain, L1 protein is detectable in four distinct molecular forms with sizes of 200, 180, 140, and 80 kDa (16). It was recently shown that L1 is processed by ADAM10 into 85- and 32-kDa fragments (17), but also the proprotein convertase PC5A plays an essential role in L1 processing leading to processed fragments of 180- and 140-kDa sizes (18).

Based on cross-reactivity of L1 antibodies, the close homologue of L1, termed CHL1, was cloned (19). In brain extracts, CHL1 is detectable in three distinct fragments of 185, 165, and 125 kDa. The 185-kDa fragment was only weakly detectable in non-detergent soluble brain extracts indicating that this might be the membrane-spanning form of CHL1, whereas the 165- and 125-kDa fragments are considered as proteolytically released fragments (20). The soluble forms of L1 and CHL1, in which the extracellular portion was fused to an Fc tail, promote neuronal survival and control neurite outgrowth (21). As demonstrated for processed L1, soluble forms can interact effectively with the extracellular matrix to regulate migration by interaction with the integrin αvβ3 (17, 22) or by unknown ligands. Similar observations have been made for CHL1 (23).

Expression of CHL1 in injured and regenerating peripheral and central neurons is strongly up-regulated (24, 25), but its functional role in regeneration remains to be defined. Because specific proteolytic processing of CHL1 may be of functional importance for neuronal survival, neurite outgrowth, and cell migration, we screened for the ability of several members of the family of ADAM metalloproteases to process CHL1 into its naturally occurring fragments.

**MATERIALS AND METHODS**

**Animals**—Breeding and experimental use of mice were performed in agreement with the German law on the protection of animals, with a permit by the local authorities. The w+ mutation was maintained on a C57BL/6 background. 30-day-old mutant mice with a manifest WR phenotype were used for biochemical analysis. Mice deficient in ADAM8 were cloned from Dr. B. M. Jockusch (Braunschweig, Germany). For purification of ADAM8 protease, COS-7 supernatants were collected 36 h after addition of serum-free Dulbecco modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum and 1% glutamine. Transient transfections were performed with LipofectAMINE (Invitrogen, Groningen, Netherlands). Stable transfected CHOK1 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% fetal calf serum, 1% glutamine, 1% penicillin-streptomycin, and 10% non-essential amino acids. From these cells, Fc fusion proteins were prepared as described previously (21).

**Western Blot Analysis**—The supernatants of transiently transfected cells were collected after addition of serum-free Dulbecco’s modified Eagle’s medium 36–48 h before harvest and were concentrated -10-fold by using Amicon Ultra Centrifugal Filter Devices 10K (Millipore, Eschborn, Germany). In order to obtain cell lysates, cells were lysed in radioimmunoprecipitation assay buffer (1× phosphate-buffered saline, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing Complete™ EDTA-free inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany) and 10 mM OPT. After incubation on ice for 1 h, samples were sonicated. Freshly prepared tissues were homogenized in radioimmune precipitation assay buffer containing the inhibitor mixture. After incubation on ice for 1 h, samples were centrifuged for 5 min at 13,000 × g at 4 °C. Supernatants were used for analysis. All samples were concentrated by affinity chromatography with ConA-Sepharose (Amersham Biosciences, Braunschweig, Germany). Proteins bound to ConA-Sepharose were washed twice with 20 mM Tris-HCl (pH 7.4), 0.5 mM NaCl, 1 mM MnCl2, 1 mM CaCl2, and were eluted with 1× SDS-PAGE loading buffer (for reducing conditions: 60 mM Tris-HCl, pH 6.8, 2% SDS, 9% glycerol, 0.0025% Bromphenol Blue, and 0.36 mM mercaptoethanol; for non-reducing running conditions, mercaptoethanol was omitted). Where indicated, cell supernatants were subjected to high speed centrifugation for 30 min at 100,000 × g to remove released membrane vesicles. For detection of CHL1 and ADAM8 proteins, samples were subjected to a 5% and 7.5% SDS-PAGE, respectively. Samples were blotted onto nitrocellulose transfer membranes (Protran, Schleicher & Schuell, Dassel, Germany) by semi-dry electroblotting. After staining with 0.1% Ponceau S solution, proteins were analyzed by immunoblotting after blocking with 5% milk powder for 2 h. Antibodies were used in a 1:10,000 dilution (crude serum of a polyclonal anti-CHL1 antibody, 1:25 (monoclonal anti-ADAM10 antibody), 1:1,000 (polyclonal anti-ADAM8-CD antibody), and 1:1,000 (polyclonal anti-ADAM10 antibody). Detection of the proteins was performed with anti-rabbit-IgG-horseradish peroxidase (1:5,000; Sigma) or anti-mouse-IgG-horseradish peroxidase (1:5,000; ImmunonResearch). Western blotting was performed using LumiLight Plus (Roche Molecular Biochemicals) as chemiluminescent substrate. For quantification of band intensities, silver-stained gels were scanned, and intensities were determined using QuanScan software (Bio-Rad, Göttingen, Germany).

**Purification of Soluble ADAMS Catalytic Domain**—COS-7 cells were transiently transfected with expression vectors pA8MPSecTag (Invitrogen, Braunschweig, Germany). Transient transfections were performed with LipofectAMINE (Invitrogen), Groningen, Netherlands). Stable transfected CHOK1 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% fetal calf serum and 1% glutamine. Transient transfections were performed with LipofectAMINE (Invitrogen) and 10% non-essential amino acids. From these cells, Fc fusion proteins were prepared as described previously (21).
by SDS-PAGE, concentrations were determined using the BCA reagent (Pherbio Science, Bonn, Germany), and proteolytic activity was monitored by a fluorescent cleavage assay in which ADAM8 cleaves the quenched fluorescent peptide Suc-H-M(cpe)-GLPLQKSH-K(Dpa)-R-amidine (27).

**Protease Assays**—Purified ADAM8 was kept in 20 mM Tris-Cl (pH 7.4) in the presence of 5 mM CaCl₂, 100 mM ZnCl₂, 100 mM NaCl, Complete EDTA-free inhibitor mixture (Roche Molecular Biochemicals). For inhibition experiments, ADAM8 was preincubated with 10 nM 1,10-orthophenanthroline (OPT) or 200 nM batimatrin (BB-94) or 500 nM of each TIMP for 1 h before substrates were added. Cleavage assays were usually performed with 1 µg of recombinant Fc-tagged proteins L1 (L1-Fc), CHL1 (CHL1-Fc), or NCAM (NCAM-Fc), described in Ref. 21, and were mixed with 1 µg of purified ADAM8 protease. Samples were incubated 3 h to overnight at 37 °C. Cleavage products were analyzed by 5% SDS-PAGE and either silver stained (28) or blotted for immunostaining with anti-Bi-Pro (for ADAM8) and anti-CHL1.

**Analysis of Protease Activities in Cell Lysates**—24 h after transfection of Chinese hamster ovary or COS-7 cells with the constructs encoding full-length ADAM proteases, the cells were washed 1× with phosphate-buffered saline and lysed with a buffer containing 20 mM HEPES, pH 7.4, 1% Nonidet P-40, and 0.5% deoxycholate. After centrifugation at 15,000 × g for 2 min, the resulting cell pellet containing cell-bound proteases was directly assayed for protease activity by adding 150 µl of mixture consisting of 50 µM TNFα substrate (Dnp-SPLAQAVERRSSSH NH₂), 1× protease inhibitor mixture in fluorescamine assay buffer (0.05 M HEPES, pH 7.4, 0.2 M NaCl, 0.01 M CaCl₂, 0.01% Brij 35). After 1–2 h at 37 °C, 25 µl of the sample was removed and mixed with 5 µl of 1% fluorescamine in Me₂SO and 70 µl of fluorescamine assay buffer. About 50 µl of this mixture was diluted with 150 µl of assay buffer, and fluorescence (excitation: 386 nm; emission: 477 nm) was measured in a PerkinElmer Life Sciences LS50B Luminescence spectrometer in black-coated 96-well plates (Nunc, Wissembourg, Germany). For activity measurements with the MBP substrate, the cell pellets were mixed with 50 µl of 100 µM MBP substrate (Suc-H-M(cpe)-GLPLQKSH-K(Dpa)-R-amidine (26)) and 50 µl of assay buffer (20 mM HEPES, pH 7.4, and 0.0015% Brij 35). Activity was measured at 340-nm excitation and 405-nm emission.

**N-terminal Sequence Analysis**—For preparation of samples for sequence, ~10 µg of CHL1-Fc protein was incubated overnight with 10 µg of soluble, recombinant ADAM8. The samples were run on an SDS-PAGE gel and washed in borate buffer (50 mM H₃BO₃, pH 9.0, 20% ethanol, 1 mM dithiothreitol) after electrophoresis. Samples were electrobotted onto polyvinylidene difluoride membranes (Porablot, Macherey Nagel, Düren, Germany) and stained with 0.2% Coomassie Brilliant Blue in 45.5% EtOH, 9% acetic acid, and 1 mM dithiothreitol. For N-terminal sequencing, the respective bands were excised and applied to a protein sequencer (Knauer, Germany). Five cycles of automated Edman degradation were run to obtain sufficient sequence information.

**Neurite Outgrowth Assays**—Cerebellar granule neurons were prepared from 6- to 8-day-old CD1 mice as described previously (29). Cells were seeded onto poly-L-lysine (PLL)-coated coverslips and were maintained in serum-free medium. The cells were washed 1× with phosphate-buffered saline and lysed with a buffer containing 20 mM HEPES, pH 7.4, 1% Nonidet P-40, and 0.5% deoxycholate. After centrifugation at 15,000 × g for 2 min, the resulting cell pellet containing cell-bound proteases was directly assayed for protease activity by adding 150 µl of mixture consisting of 50 µM TNFα substrate (Dnp-SPLAQAVERRSSSH NH₂), 1× protease inhibitor mixture in fluorescamine assay buffer (0.05 M HEPES, pH 7.4, 0.2 M NaCl, 0.01 M CaCl₂, 0.01% Brij 35). After 1–2 h at 37 °C, 25 µl of the sample was removed and mixed with 5 µl of 1% fluorescamine in Me₂SO and 70 µl of fluorescamine assay buffer. About 50 µl of this mixture was diluted with 150 µl of assay buffer, and fluorescence (excitation: 386 nm; emission: 477 nm) was measured in a PerkinElmer Life Sciences LS50B Luminescence spectrophotometer in black-coated 96-well plates (Nunc, Wissembourg, Germany). For activity measurements with the MBP substrate, the cell pellets were mixed with 50 µl of 100 µM MBP substrate (Suc-H-M(cpe)-GLPLQKSH-K(Dpa)-R-amidine (26)) and 50 µl of assay buffer (20 mM HEPES, pH 7.4, and 0.0015% Brij 35). Activity was measured at 340-nm excitation and 405-nm emission.

**RESULTS**

**Cleavage of Recombinant CHL1 by ADAM8**—Recombinant ADAM8 was expressed as a secreted soluble form from COS-7 cells containing the pro- and metalloprotease domain (A8-MP). To monitor for catalytic activity, a parallel assay with a fluorogenic substrate was performed (27). Extracellular domains of NCAM, L1, and CHL1 (Fig. 1) fused to an Fc tag (21) were incubated with purified ADAM8 (Fig. 2A). Whereas NCAM and L1 were not cleaved by ADAM8, CHL1-Fc was cleaved into two products: a prominent 165-kDa fragment corresponding to the entire extracellular portion of CHL1 and a less prominent
125-kDa fragment. Whereas the 165-kDa fragment was detectable under reducing and non-reducing conditions, the 125-kDa fragment disappeared under non-reducing conditions (Fig. 2 A, right panel). Specificity of the cleavage was confirmed by immunostaining using an antibody against the extracellular portion of CHL1 (Fig. 2 B), and equal loading as well as processing of pro-ADAM8 into active protease by autocatalytic prodomain removal was confirmed by Western blot analysis (Fig. 2 C). Usually, about 50% of the ADAM8 protein is processed into the active form. In Western blot experiments with anti-CHL1, the specific cleavage of CHL1 was confirmed by detecting the uncleaved CHL1-Fc protein of about 200-kDa size and besides the cleaved CHL1 fragments, bands of 80 kDa and 40 kDa, corresponding to the fragments of CHL1-Fc containing the residual Fc portion (Fig. 2 B), which were subjected to N-terminal sequencing. For the 165-kDa fragment, the cleavage site was determined at LVP\_GAEHIV located within the fifth partial FN III-like domain at amino acid position 1040, for the 125-kDa fragment, the cleavage site within the second FN III-type domain was determined at amino acid position 753 in the sequence WKP\_QGAPE (Fig. 1). Extensive cleavage of CHL1 by soluble ADAM8 was inhibited by broad-range inhibitors of metalloproteases, BB-94 and OPT, but not by 500 nM TIMPs 1–4, as demonstrated by a silver-stained gel and by quantitative analysis (Fig. 2 D). The observed inhibition profile is in accordance with previous cleavage studies on ADAM8 (27, 31).

**Fig. 2. Cleavage analysis of CHL1-Fc, NCAM-Fc, and L1-Fc by recombinant ADAM8.** A, recombinant Fc fusion proteins CHL1-Fc, NCAM-Fc, and L1-Fc were used; from each protein, 1 μg was subjected to cleavage with 1 μg of affinity-purified soluble ADAM (A8MP) at 37 °C for 3 h. Samples were run on a 5% gel and silver-stained. M, molecular weight standard (M, left). For NCAM-Fc and L1-Fc, no cleavage was observed; in contrast, cleavage of CHL1-Fc resulted in a predominant 165-kDa fragment and, to less extent, a 125-kDa fragment (left arrows). For each recombinant protein, three lanes are shown with only protein in cleavage buffer (−A8-MP), with ADAM8 (+A8-MP), and with ADAM8 in the presence of 200 nM batimastat (+A8-MP/BB-94), respectively. In the right panel, cleavage of CHL1 is shown under reducing (+MeSH) and non-reducing conditions (−MeSH). Note that under non-reducing conditions, the 125-kDa band (marked by an asterisk) is absent; B, Western blot analysis of CHL1 cleavage by ADAM8. Conditions were identical to those described in A; the blot was stained with rabbit anti-CHL1 antibody (1:10,000). Relevant fragments are indicated by arrows, and the double arrow indicates the 125-kDa and the complementary 80-kDa fragments. C, immunodetection of ADAM8 protease in purified supernatants from COS-7 cells. Pro-ADAM8 (pro-A8MP) with a molecular mass of 56 kDa, processed ADAM8 (A8-MP) with a molecular mass of 29 kDa. D, quantitative analysis of CHL1-Fc cleavage in the presence of ADAM inhibitors 1,10-ortho-phenanthroline (OPT, 10 mM), BB-94 (200 mM), and TIMPs 1–4 (500 nM). The cleavage reactions are shown in the diagram in the same order as below. For each condition, band intensities of uncleaved CHL1-Fc protein was determined and compared with 1 μg of uncleaved CHL1-Fc protein (−100%), given as means ± S.E.
we performed fluorescence activity assays with cells transiently transfected with full-length cDNAs for ADAM8, EQ-ADAM8, ADAM10, and ADAM17 (Fig. 3A). In cell lysates from these cells, we determined protease activities for the MBP peptide substrate with ADAM8 and ADAM10, but not with lysates from mock transfected cells, EQ-ADAM8, and ADAM17. To monitor ADAM17 activity, we used the TNF-α substrate, which is significantly cleaved only by ADAM17. These data indicate that the mouse cDNAs used to express ADAM8, ADAM10, and ADAM17 encode functional proteases in COS-7 cells.

Cleavage of CHL1 by ADAM8 in Cell Culture—To detect ectodomain shedding of CHL1 by ADAM8 in cell culture, full-length CHL1 and ADAM8 were co-transfected into COS-7 cells. Cell lysates and supernatants were analyzed. For comparison, wild type ADAM8 and the catalytically inactive ADAM8, in which Glu330 was exchanged by Gln (EQ-ADAM8 (27)) were also co-transfected. The full-length 185-kDa CHL1 transmembrane protein was detected, slightly more in cells transfected with EQ-ADAM8 than in cells transfected with ADAM8. Co-transfection of ADAM8 significantly increased the amount of soluble 165- and 125-kDa fragments of CHL1 in supernatants of cells transfected with ADAM8, but not with EQ-ADAM8 (Fig. 3B).

We conclude from this experiment that ADAM8 expression in CHL1-transfected COS-7 cells results in significantly higher amounts of soluble CHL1 compared to the basal CHL1 shedding. The specificity of the cleaving protease is demonstrated by co-transfection experiments with cDNAs for CHL1 and ADAM10 or CHL1 and ADAM17, in which no significant cleavage of CHL1 was observed. Using high speed centrifugation of the supernatant, the ~60-kDa band representing the membrane stub and the corresponding 125-kDa band were removed; also under non-reducing conditions, the 125-kDa band was not detectable (Fig. 3B). To analyze the dependence of ADAM8-mediated shedding on activators of protein kinase C, well described for ADAM17/TACE, we performed co-transfection experiments in the presence of phorbol ester TPA and pervanadate, a phosphatase inhibitor. No significant effect on CHL1 shedding was observed, even when TPA and pervanadate were applied in high concentrations (Fig. 3C).

CHL1 Shedding in ADAM8-deficient Mice—The correlation between ADAM8 expression and CHL1 shedding was further analyzed in situ. Proteolytic processing of CHL1 in brain extracts from 30-day Wobbler (WR) mutant mice and from mice deficient in ADAM8 was compared with wild type mice (Fig. 4). In cerebellum/brain stem from wild type mice, ADAM8 is present in its catalytically active form, and CHL1 processing into the 125- and 165-kDa fragments was observed. In cerebellum/brainstem from Wobbler mice, an up-regulation of ADAM8 has been demonstrated as a consequence of neurodegeneration and astrogliosis (13) (Fig. 4A). Consistently, an increased proteolytic processing of 185-kDa CHL1 into both soluble fragments was detected (Fig. 4A). In homozygous ADAM8-deficient mice, the ADAM8 protein was not detectable. Processing of CHL1 in brain extracts of these mice was dramatically reduced emphasizing a significant contribution of ADAM8 to the proteolytic processing of CHL1 in this particular brain region in situ.

Effect of CHL1 Shedding on Neurite Outgrowth in Neuronal Cell Cultures—To analyze the physiological function of soluble CHL1 generated by ADAM8-dependent ectodomain shedding, we performed neurite outgrowth and cell survival assays with mouse cerebellar neurons in the presence of conditioned media from CHL1 and ADAM8 co-transfected COS-7 cells (Fig. 5). As described previously, recombinant L1-Fc or CHL1-Fc proteins are able to stimulate neurite outgrowth from cerebellar or hippocampal neurons (21). Supernatants from COS-7 cells transfected with ADAM8, CHL1, or both were derived by 24-h maintenance of cells in serum-free medium. Supernatants from ADAM8- or CHL1-transfected cells showed a basal level of neurite outgrowth (20 ± 1.6 μm for ADAM8, 22 ± 1.9 μm for CHL1). In contrast, supernatants from cells co-transfected with ADAM8 and CHL1 caused significantly increased neurite outgrowth up to 43 ± 4 μm compared with only CHL1 transfected cells (22 ± 2 μm). A comparable rate to the basal level of neurite outgrowth was achieved with EQ-ADAM8/CHL1 (21 ± 2 μm) or ADAM10/CHL1 (19.5 ± 2.5 μm) or ADAM17/CHL1 (21 ± 3 μm). In the same experiments, recombinant CHL1-Fc protein was used as a standard. In a dose-response curve, 1 μg/ml CHL-Fc protein resulted in a maximal neurite outgrowth (45 ± 5 μm). By a dot blot assay, we determined the concentration of the soluble CHL1165 fragment by comparison with known CHL1-Fc concentrations and determined a concentration of ~100 ng/ml soluble CHL1 in the supernatant. Based on this calculation we assume that the concentration of soluble CHL1 required for the maximal neurite outgrowth is one order of magnitude lower than the concentration of CHL1-Fc fusion protein. We can not exclude that soluble CHL1 may attach to the substrate and exerts its effect not only as a soluble molecule, but this also holds for CHL1-Fc, so the effects may be comparable.

Enhancement of Neurite Outgrowth by Substrate-coated Soluble CHL1—We used a co-culture of cerebellar granule neurons with transfected COS-7 cells to determine if CHL1 proteolysis enhances neurite outgrowth when offered as a substrate (Fig. 6). The numbers of substrate-attached cells as well as the neurite lengths were determined. In accordance with the previous experiment, the number of substrate-attached cells was significantly larger when granule neurons were plated together with COS-7 cells transfected with ADAM8 and CHL1 (375 ± 20). A lower number of substrate-attached cells and significantly lower levels of neurite outgrowth were observed when either ADAM8 or CHL1 were used alone (100 ± 5 and 180 ± 7, respectively), or when ADAM10 (160 ± 10) and ADAM17 (168 ± 3) were used. Instead of EQ-ADAM8, we used batimastat (BB-94) for inhibition of CHL1 cleavage in CHL1/ADAM8 co-transfected cells (170 ± 13). When shedding of CHL1 was inhibited by BB-94, substrate attachment was reduced below the basal level obtained with CHL1 alone. This difference suggests that, in addition to ADAM8, another unknown protease that is inhibited by BB-94 could be involved in ectodomain shedding of CHL1, although at a much lower level.

Reduction of Cell Death by Soluble CHL1—The time course of cell death of cerebellar granule neurons was determined by using serum-free medium from COS-7 cells transfected either with CHL1 alone or with CHL1/ADAM8 (Fig. 7). As estimated by the trypan blue exclusion method, the number of surviving cells declined to 19% ± 1.5% after 5 days in culture when the conditioned medium was derived from either only CHL1 or CHL1/EQ-A8 transfected cells. Similar values were obtained when neurons were treated with supernatants from CHL1/ADAM10 and CHL1/ADAM17 co-transfected cells (data not shown). In contrast, a significantly higher proportion (36% ± 1.8%) of cells survived when conditioned medium from COS-7 cells co-transfected with ADAM8 and CHL1 was applied to neuronal cultures. Thus, survival of neurons was increased by ~50% due to the presence of soluble CHL1 derived by proteolytic processing.

DISCUSSION

We present the first evidence that ectodomain shedding of the neural cell adhesion molecule CHL1 by the metalloprotease-disintegrin ADAM8 has a physiological role by providing
soluble CHL1, which is able to promote neurite outgrowth and reduce cell death of cerebellar neurons. CHL1 cleavage by ADAM8 is specific, because neither ADAM10 nor ADAM17, both expressed in brain in significantly higher levels than ADAM8, were able to cleave CHL1. In addition, L1-Fc and NCAM-Fc fusion proteins were not cleaved by ADAM8 suggesting a defined substrate-protease relationship. Given the fact that the metalloprotease domain of ADAM8 was used for our studies, we conclude that other domains of ADAM8 are not essential for the observed substrate specificity. It is interesting to note that, in addition to cleavage in the fibronectin type III homologous domain (FN III) 2 (position 753, WKP | GQAPE), the main cleavage site in the CHL1 protein is within the fifth FN domain of CHL1 in the juxtamembranous region (position 1040, LVP | GAEHI). Under non-reducing conditions, the 125-kDa fragment of CHL1 was hardly detectable, suggesting that the cleavage site generating this fragment is located between two cysteine residues forming a disulfide bond under native conditions. This argues for the membrane proximal region to contain the physiologically relevant cleavage site of CHL1 for the release of the extracellular domain rather than the membrane distal one. The fifth FN III domain is not present in other related immunoglobulin superfamily members and has the lowest homology between L1 and CHL1, which could explain the clear-cut substrate specificity. It may be interesting to note that a weak similarity of the 165-kDa CHL1 cleavage site was found for the autocatalytic cleavage site of ADAM8 (40% within a 10-amino acid stretch from P5 to P5: ADAM8, NDLGP | RALEI; CHL1, EVLVP | GAEHI). Experiments to introduce point mutations or a 10-amino acid deletion into the 165-kDa cleavage site of CHL1 did not provide sufficient information about critical amino acid residues, because the observed deficit in proteolytic cleavage is most likely due to the finding that mutated CHL1 proteins did not reach the cell surface. Thus the physiological consequences of the ADAM8-mediated proteolytic cleavage of CHL1 could not be investigated.

In cell-based assays, release of the extracellular domain of CHL1 by ADAM8 was not enhanced by activators of ectodomain shedding such as protein kinase C activators, for instance the phorbol ester TPA or by the phosphatase inhibitor pervanadate. This is in contrast to numerous reports on activation of TACE/ADAM17-induced ectodomain shedding by these compounds, suggesting that the mechanisms of intracellular regulation of proteolytic activity for ADAM8 is different from that of ADAM17 involving protein kinase C epsilon (32).

In neurite outgrowth stimulation experiments, soluble CHL1 was 10 times more potent than the CHL1-Fc protein. This observation supports the notion that proteolytic release of CHL1 is of high functional relevance in situ. Release of CHL1 into the extracellular milieu would allow the molecule to diffuse away from the cell surface and to bind to receptor(s) remote from the original site of CHL1 synthesis. The one or more receptors have so far remained elusive.

A role for CHL1 in cell migration has recently been demonstrated (23). In this study, α5β1 and α6β1 integrin-mediated cell migration toward collagen I was enhanced by CHL1. Similar to L1, integrin-dependent cell migration could be potentiated by fragments released from membrane-bound CHL1 (22). Thus, ADAM8 may have multiple effects on cell adhesion, either by cleavage of CHL1 or by direct integrin binding via its disintegrin domain (1, 7, 77 | H11003, 770 | H11003, 77 | H11003). 2

### Fig. 3. Cleavage of CHL1 by ADAM8 in COS-7 cells.

| Treatment          | Relative Fluorescence (M) |
|--------------------|---------------------------|
| Mock               | 0.5                       |
| ADAM8              | 2.5                       |
| EQ-ADAM8           | 3.0                       |
| ADAM10             | 2.8                       |
| ADAM17             | 2.6                       |

**A** cell-bound protease activity in COS-7 after transient transfections, given as relative mean fluorescence ± S.E. after a 1-h reaction. Mock, pTarget without insert. For ADAM8, EQ-ADAM8, and ADAM10, the fluorescent MBP peptide was used; for ADAM17 the TNF-α peptide was used. B, co-transfection of CHL1/wild-type ADAM8 and CHL1/mutant EQ-ADAM8 in COS-7 cells. In the cell lysate (upper panel), the unprocessed 185-kDa form of CHL1 is detectable with CHL1 antibody. In serum-free media of cells co-transfected with A8 and CHL1, 165-kDa and 125-kDa fragments and the membrane stub (~60 kDa) were detected by CHL1 antibody (middle panel). Co-transfection of CHL1 and EQ-A8 (Glu305 to Gln exchange in the catalytic site) did not result in CHL1 shedding (right lane). In the right panel, co-transfections with ADAM10 and ADAM17 did not result in significant cleavage of CHL1. In addition, the supernatants of A8/CHL1-transfected cells were analyzed after a high speed spin ("spin") and under non-reducing ("nr") conditions without mercaptoethanol. Note that under both conditions, the 125-kDa band as well as the membrane stub fragment are absent. A8 and EQ-A8 and ADAM17 were detected by anti-Bi-Pro antibody 4A6 recognizing a tag in the C terminus of the proteins. For ADAM10, we used a polyclonal serum against the cytoplasmic tail. **White arrowheads** indicate proforms; **filled arrowheads** indicate processed forms of ADAM proteins. C, shedding experiments in the presence of commonly used activators TPA (50 and 500 ng/ml) and pervanadate (1×, 77 μM; 10×, 770 μM); "ctrl", co-transfection of ADAM8 and CHL1 without activators. One day after transfection, medium was changed to serum-free conditions in the absence or presence of the activators. 6 h later supernatants were harvested.
grin domain (27) thus activating intracellular signal transduction pathways in coordination with CHL1.

In the central nervous system, ADAM8 and CHL1 are expressed in neurons and oligodendrocytes (19, 20) so that processing of CHL1 by ADAM8 in these cells could occur in cis interaction on the same cell surface. Whether ADAM8 interacts directly or indirectly with CHL1 remains to be analyzed. An interesting possibility is the interaction of ADAM8/integrins with CHL1.

**Fig. 4. Cleavage of CHL1 in WR mutant and ADAM8-deficient mice.** Brain homogenates from 30-day-old wild type C57BL/6 mice, age-matched homozygous Wobbler mutant mice (A) and homozygous ADAM8-deficient mice (B) were prepared from cerebellum/brain stem, and 20 µg of extracts or ConA eluates was separated on 5% gels. Blots were probed with anti-CHL1 antibody and with anti-glyceraldehyde-3-phosphate dehydrogenase as loading control. For ADAM8, ConA preparations were used and stained with A8 antibody directed against the cytoplasmic domain of ADAM8. In A, upper panel, a short exposure of the blot showing intensities of 185- and 165-kDa bands, respectively. In the longer exposure (below), cleavage of the 125-kDa band was enhanced in WR mice. In B, brain extracts from wild type mice (+/+) and ADAM8-deficient mice (−/−) were probed for CHL1 (upper panel), ADAM8 (middle panel), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Fig. 5. Effect of supernatants from COS-7 cells on neurite outgrowth.** Cerebellar granule neurons were treated with supernatants obtained from COS-7 cells transfected with ADAM8, CHL1, ADAM8/CHL1, EQ-A8/CHL1, ADAM10/CHL1, and ADAM17/CHL1 for 18 h after transfections. Protease expression was determined in COS lysates (lys) as described in Fig. 3. In the diagram, neurite outgrowth was quantified by determining the neurite lengths of at least 50 individual neurons after staining with Richardson's blue. Values are from four independent experiments and given as the means ± S.E. Below, COS-7 supernatants (SN) analyzed with CHL1 antibody to detect the 165-kDa soluble form of CHL1 and corresponding micrographs showing neurite outgrowth of cerebellar granule neurons (CGN) in an area stained with neurofilament (NF 200) antibody.

**Fig. 6. Effect of CHL1 shedding on neuronal survival and neurite outgrowth in co-culture with COS-7 cells.** COS-7 were transfected with constructs encoding the indicated proteins. 12 h after transfection, cerebellar neurons were plated on top of COS-7 cells. BB-94 (200 nM) was added 6 h after transfection and was repeatedly added after 6 h. A, the number of attached neurons (with neurite lengths longer than their diameter) was determined in at least 50 randomly chosen visual fields by phase-contrast microscopy, and values are given as cell numbers; B, average neurite length was determined similar to data presented in Fig. 5. Values are from three independent experiments in triplicates and given as the means ± S.E.

**Fig. 7. Effect of CHL1 on cell death.** Death of granule neurons in the presence of either supernatant from CHL1 transfected, CHL1/ADAM8, and CHL1/EQ-A8 co-transfected COS-7 cells at different culture times in serum-free medium. The numbers of living cells were determined by trypan blue exclusion. Values are from three independent experiments in triplicates and are given as the means ± S.E. Asterisks denote significant differences from neurons cultured with CHL1 alone. ***, p < 0.001; **, p < 0.01 (p < 0.05 by Student’s t test).
with CD9 (tetraspanin), which would recruit CHL1 into a complex, in a mechanism similar to that discussed for the regulation of shedding of heparin-binding epidermal growth factor HB-EGF (33). Interestingly, under pathological conditions, ADAM8 is additionally expressed by astrocytes and microglia (13), and, for reactive astrocytes in the optic nerve, enhanced CHL1 expression was also reported (25). Thus, cleavage of CHL1 under pathological conditions could result in enhanced remodeling of reactive astrocytes extending their processes, a hallmark of many neuropathological states.

Based on the enhanced expression of ADAM8 in Wobbler mice, it is likely that increased CHL1 shedding under pathological conditions is an important event in the reactive activation of glial cells in the central and possibly in the peripheral nervous system. In addition, the proinflammatory cytokine TNF-α is an important signaling molecule in diseases of the central nervous system (34), where TNF-α induces expression of ADAM8 (13), which in turn may lead to enhanced proteolytic release of CHL1, as observed in the Wobbler mouse. In the cerebellum and brain stem of Wobbler mice, enhanced cleavage but not transcriptional up-regulation of CHL1 mRNA expression was observed. Thus, it is likely that TNF-α regulates CHL1 shedding via transcriptional activation of ADAM8. It remains to be determined whether enhanced CHL1 shedding in situ has similar effects as in cell culture particularly regarding support of axonal regeneraiton and suppression of neuronal cell death after trauma.

Acknowledgments—We thank Elke Redecker and Angela Perz for excellent technical assistance; Dr. Uwe Schloemann for providing ADAM8, ADAM10, and ADAM17 constructs; Dr. Harald Tchesche for the kind gift of recombinant TIMPs and BB-94 and Dr. Olaf Kruse for help with fluorescence measurements; Kerstin Bo¨cker (Department of Biochemistry) for N-terminal sequencing, and Dr. Tiebang Kang (Florida State University, Tallahassee) for helpful comments on the manuscript.

REFERENCES
1. Peschon, J. J., Slack, J. L., Reddy, P., Stoecking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlosky, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J., and Black, R. A. (1997) Science 282, 1281–1284
2. Seals, D. F., and Courtneidge, S. A. (2003) Trends Genet. 19, 7–30
3. Bolk, C. P. (2000) Curr. Opin. Cell Biol. 12, 606–612
4. Primakoff, P., and Myles, D. G. (2000) Trends Genet. 16, 83–87
5. Chantry, A., Gregson, N. A., and Glynn, P. (1989) J. Biol. Chem. 264, 21683–21687
6. Moss, M. L., Jin, S. L., Mills, E. A., Bickett, D. M., Burkhart, W., Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J. L., and Becker, J. D. (1997) Nature 385, 723–736
7. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stoecking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Bosani, N., Schoelen, K., Gerhart-Hines, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) Nature 385, 729–733
8. Brea, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doreens, J. R., Cumano, A., Roux, P., Black, R. A., and Israel, A. (2000) Mol. Cell 5, 207–216
9. Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C., and Lee, D. C. (2003) EMBO J. 22, 2784–2796
10. Iwamoto, R., Yamaizaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G., Nanba, D., Higashiya, S., Horii, M., Klagsbrun, M., and Mekada, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3221–3226
11. Buxbaum, J. D., Liu, K. N., Luo, Y., Black, J. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P., and Black, R. A. (1998) J. Biol. Chem. 273, 27765–27767
12. Yoshida, S., Setoguchi, M., Higuchi, Y., Akizaki, S., and Yamamoto, S. (1990) Int. Immunol. 2, 585–591
13. Schloemann, U., Rathi-Hartlieb, S., Yamamoto, S., Jockusch, H., and Bartsch, J. W. (2000) J. Neurosci. 20, 7964–7971
14. Fourie, A. M., Coles, F., Moreno, V., and Karlsson, L. (2003) J. Biol. Chem. 278, 30469–30477
15. Rathjen, F. G., and Schachner, M. (1986) EMBO J. 5, 1–10
16. Sadoul, K., Sudol, R., Faissner, A., and Schachner, M. (1988) J. Neurochem. 50, 510–521
17. Mechtner, S., Gutwein, P., Aegon-Levin, N., Stoeck, A., Oleszewski, M., Riedle, S., Postina, R., Fabriczho, P., Vogel, M., Lemmon, V., and Allevogt, P. (2001) J. Cell Biol. 155, 661–673
18. Kalaus, I., Schneegiesberg, B., Siebold, N. G., Kleene, R., and Schachner, M. (2001) J. Biol. Chem. 276, 10391–10396
19. Holm, J., Hillenbrand, R., Steuber, V., Bartsch, U., Mos, M., Kubbert, H., Montag, D., and Schachner, M. (1996) Eur. J. Neurosci. 8, 1613–1629
20. Hillenbrand, R., Molihr, M., Montag, D., and Schachner, M. (1999) Eur. J. Neurosci. 11, 813–821
21. Chen, S., Mantei, N., Deng, L., and Schachner, M. (1999) J. Neurobiol. 38, 428–439
22. Tauss, K., Kedar, V., Panicker, A. K., Schmid, R. B., and Maness, P. F. (2002) J. Neurosci. 22, 4918–4931
23. Buhusi, M., Midkiff, B. R. Gates, A. M., Richter, M., Schachner, M., and Maness, P. F. (2003) J. Biol. Chem. 278, 25024–25031
24. Chaisukont, V., Zhang, Y., Anderson, P. C., Campbell, G., Vaudano, E., Schachner, M., and Lieberman, A. R. (2000) Neuroscientist 100, 87–108
25. Rolf, B., Lang, D., Hillenbrand, R., Richter, M., Schachner, M., and Bartsch, U. (2003) J. Biol. Chem. 278, 835–843
26. Riediger, M., Jockusch, B. M., and Rothkugel, M. (1997) BioTechniques 23, 96–97
27. Schloemann, U., Wildeboer, D., Webster, A., Astropovka, O., Zeuschner, D., Dockeby, A. J., McLaughlin, S., Skelton, L., Jockusch, H., and Bartsch, J. W. (2002) J. Biol. Chem. 277, 48210–48219
28. Blum, H., Beier, H., and Gross, H. J. (1987) Electrophoresis 8, 93–99
29. Kreilbauer, G., Faissner, A., and Schachner, M. (1985) Nature 316, 729–730
30. Fischer, G., Kunemund, V., and Schachner, M. (1986) J. Neurosci. 6, 605–612
31. Amour, A., Knight, C. G., Douglas, W. A., Roux, P., Black, R. A., Bartsch, J. D., Blom, C. P., and Murphy, G. (2002) FEBS Lett. 524, 154–158
32. Wheeler, D. L., Ness, K. J., Oberley, T. D., and Verma, A. K. (2003) Cancer Res. 63, 6547–6555
33. Zhang, X. A., Bontrager, A. L., and Hemler, M. E. (2001) J. Biol. Chem. 276, 25005–25013
34. Szelenyi, J. (2001) Brain Res. Bull. 54, 329–338
Ectodomain Shedding of the Neural Recognition Molecule CHL1 by the Metalloprotease-disintegrin ADAM8 Promotes Neurite Outgrowth and Suppresses Neuronal Cell Death

Silvia Naus, Melanie Richter, Dirk Wildeboer, Marcia Moss, Melitta Schachner and Jörg W. Bartsch

*J. Biol. Chem.* 2004, 279:16083-16090.
doi: 10.1074/jbc.M400560200 originally published online February 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400560200

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 34 references, 15 of which can be accessed free at http://www.jbc.org/content/279/16/16083.full.html#ref-list-1