Collapsin Response Mediator Proteins of Neonatal Rat Brain Interact with Chondroitin Sulfate*

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Chondroitin sulfate proteoglycans are structurally and functionally important components of the extracellular matrix of the central nervous system. Their expression in the developing mammalian brain is precisely regulated, and cell culture experiments implicate these proteoglycans in the control of cell adhesion, neuron migration, neurite formation, neuronal polarization, and neuron survival. Here, we report that a monoclonal antibody against chondroitin sulfate-binding proteins from neonatal rat brain recognizes collapsin response mediator protein-4 (CRMP-4), which belongs to a family of proteins involved in collapsin/semaporin 3A signaling. Soluble CRMPs from neonatal rat brain bound to chondroitin sulfate affinity columns, and CRMP-specific antisera co-purified chondroitin sulfate. Moreover, chondroitin sulfate and CRMP-4 were found to be localized immunohistochemically in overlapping distributions in the marginal zone and the subplate of the cerebral cortex. CRMPs are released to culture supernatants of NTera-2 precursor cells and of neocortical neurons after cell death, and CRMP-4 is strongly expressed in the upper cortical plate of neonatal rat when cell death is abundant. Therefore, naturally occurring cell death is a plausible mechanism that targets CRMPs to the extracellular matrix at certain stages of development. In summary, our data indicate that CRMPs, in addition to their role as cytosolic signal transduction molecules, may subserve as yet unknown functions in the developing brain as ligands of the extracellular matrix.

The extracellular matrix (ECM)1 of the developing brain has a unique composition from lecticans, tenascins, laminins, and hyaluronic acid (1–5). Lecticans are proteoglycans that carry chondroitin sulfate (CS) side chains on core proteins encompassing a N-terminal hyaluronan binding domain and a C-terminal lectin domain. Four different members of the lectican protein family are known (neurocan, aggregan, versican, and brevican) (1). Their multidomain composition enables lecticans to interact with multiple cell surface molecules and diffusible ligands (see below) (6, 7). Chondroitin sulfates are glycosaminoglycans composed of repeated glucuronic acid-[|61,3]-N-acetylgalactosamine disaccharide units that are linked by [|61,4]-glycosidic bonds into long unbranched chains with molecular masses of up to 50 kDa and more (8). With respect to the position of sulfate esters at the galactosamine, CSA (C-4-S, containing C-4-sulfate) and CSC (C-6-S, containing C-6-sulfate) are distinguished. The expression of these proteoglycan core proteins is precisely tuned during brain development (9). Moreover, the disaccharide composition of CS is regulated (9, 10).

The functions of chondroitin sulfate proteoglycans (CS-PGs) in neural tissue can be categorized into effects on cell adhesion, cell migration, neurite formation, neuron polarization, synaptic modulation/plasticity, and neuron survival (for reviews, see Ref. 6 and references therein and Ref. 11). These effects often critically depend on glycosaminoglycans or may even be attributed solely to glycosaminoglycan chains. Many of the listed functions, however, have been discovered using in vitro experiments that were designed in a way that the proteoglycan or glycosaminoglycan component became limiting in the assays. In vivo, on the other hand, there is marked structural redundancy of ECM components (1, 3) if one considers for example the existence of four different lecticans (see above). Thus, phenotypes of knockout animals lacking a single ECM protein are often rather mild (for example in knockouts for neurocan (12) and tenasin-C (13, 14)). Inactivation of enzymes involved in CS biosynthesis, on the other hand, could lead to more severe phenotypes since CS is a component of multiple ECM proteins. In the chondroitin-6-sulfate transferase knockout mouse, however, no major CNS pathology was found (15). C-6-S may be replaced by C-4-S, and expression data suggest that C-4-S is probably more important in the developing brain. Nevertheless, the importance of CS in vivo is underscored impressively by the observation that after local treatment with chondroitinase ABC, which degrades C-4-S, C-6-S, and dermatan sulfate, regeneration of functional neurites in the adult spinal cord is enabled (16). Thus, CS-PGs are considered to contribute to the inhibition of regenerative responses in the adult mammalian nervous system.

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The current insight into the mechanisms of actions of neurons is still rudimentary. Binding partners of CS-PGs at the plasma membrane include sulfatide and several (eventually glycosylphosphatidylinositol-anchored) cell adhesion molecules of the Ig family such as N-CAM, L1, TAG-1, and F3/ contactin (for a review, see Ref. 6). The signaling events exerted after binding of CS-PGs to these molecules are unknown. On the other hand, CS-PGs bind a variety of soluble ligands including growth factors like bovine fibroblast growth factor and oligomeric glycoproteins of the ECM-like tenascins (6). Interestingly, CS is a critical component of a molecular scaffold to which diffusible molecules are bound that convey inhibitory or promoting actions, e.g., on the adhesion of thalamic neurons and the formation of neurites (17). The identity of these diffusible molecules, however, is as yet unknown.

To elucidate the molecular basis of the neurotrophic actions of chondroitin sulfate, we previously fractionated protein extracts from neonatal rat brain on a chondroitin sulfate affinity column and used the eluted binding proteins to generate monoclonal antibodies (18). One of these antibodies, termed mAb-9, recognizes a 65-kDa protein with laminar expression in the neocortex, which parallels the expression of CS. The protein is present in both the fraction of soluble proteins and in the particulate fraction of neonatal rat brain. The aim of the present study was to identify this chondroitin sulfate-binding protein and to characterize its interaction with glycosaminoglycans. We show that mAb-9 recognizes a soluble protein that is present in the cytosol, termed collapsin response mediator protein-4 (CRMP-4). This protein and its relatives interact with chondroitin sulfate, and they are released from the cytosol of neurons to the extracellular space most probably after cell death. This may explain why CRMP-4 was found to be localized with chondroitin sulfate in the developing neocortex of rat brain in regions where naturally occurring cell death is prevalent.

MATERIALS AND METHODS

Unless otherwise stated, chemicals were from Serva (Heidelberg, Germany), Sigma, Roche Molecular Biochemicals, or Merck. ZERO Blunt vector for PCR cloning was from Invitrogen, and pQE-30 vector for bacterial expression of histidine-tagged proteins was from Qiagen (Hilden, Germany). SDS PAGE and Western Blots—SDS-PAGE was performed on 5–15% gradient slab gels (Bio-Rad Protean II) or 10% mini-gels (Bio-Rad MiniProtein) (19). Gels were stained by silver (20), Coomassie Blue, or zinc/imidazole (21). For Western blotting, proteins were transferred to nitrocellulose in a semi-dry blotting apparatus (Bio-Rad) according to Kyhse-Andersen (22). After blocking with 3% nonfat dry milk powder, 1% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBST), the first antibody incubation was performed according to the manufacturer’s instructions using an oligo-dT primer. After digestion with RNase H one-tenth of the reaction was used as template for PCR amplification by Pf polymerase (Stratagene, La Jolla, CA) using 94 °C for 90 s, 55 °C for 45 s, 72 °C for 2 min for 30 cycles. The following primer pairs were used: CRMP-1(5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’)/Hind, 5’-CTATCAGGGG-3’; CRMP-2(5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’)/Hind, 5’-ATGCCTCCAAAGCAAGCAGGAGCTTCCCCCAGATA-3’; CRMP-3(5’-ACAAGAGACCCCAAGTCTAAGAAAG-3’); CRMP-4(5’-TGCCGCACCGCAAGCTTACTCAGGGATGT-3’)/Hind, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’). PCR products were gel-purified, digested with ATCGAGA-3’, CRMP-1(5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’)/Hind, 5’-TGCCGCACCGCAAGCTTACTCAGGGATGT-3’; CRMP-2(5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’)/Hind, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’; CRMP-3(5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’)/Hind, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’; CRMP-4(5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’)/Hind, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-3’). The PCR products were cloned into the ZERO Blunt vector and sequenced using the ABI Prism 310 Genetic Analyzer.

Once-overexpression of CRMPs—Bacterially overexpressed in Escherichia coli was performed using the Qiexpress kit (Qiagen) according to the instructions of the manufacturer. The coding regions of the four CRMP-cDNAs were re-amplified using primers containing restriction sites for BamHI/PEPS and HindIII and the pcPB vectors containing the CRMP inserts as templates. The following primer pairs were used: CRMP-1(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-1(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-2(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-2(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-3(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-3(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-4(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’; CRMP-4(5’/Sal, 5’-ACCTAGCGTCGACACATAGAAGGTAGAATGTC-TCTACACGCGG-3’). The PCR products were gel-purified, digested with HindIII, and ligated into the pcPB vector, thereby incorporating a 5’ extension of the cDNA coding for a His tag, tag. M15[pRep] bacteria were transformed, and ampicillin/kanamycin resistant strains were analyzed for CRMP cDNA inserts and sequenced to verify ligiation sites and the PCR products. Positive strains were grown.
in Luria Bertani medium containing kanamycin and ampicillin, and expression was induced by 2 mM isopropyl-β-D-thiogalactopyranoside. Cells were lysed in Triton/phosphate buffer containing 8 mM urea, and expression of recombinant His-tagged CRMP proteins was tested by Western blot with an anti-RGS-His antibody (Qiagen).

**Antiserum Production, Affinity Purification of Antibodies, and Biotinylation of IgG—**Antibodies raised in rabbit against the synthetic peptide, HIRLTTTPGKCTQARSACGKIS (residues 504–527), and CRMP-Fam-pee (SFYADYIEMDGLIKQGDGN, rCRMP-4 residues 30–48) were obtained from Pineda Antikörper Service, Berlin, Germany. CRMP-1-pee (YEVPATPKHAAP-SAKSSPSKQK, rCRMP-1 residues 504–527), CRMP-2-pee-a (CEVS-PKVTVPASSKTSPAKQOR, rCRMP-2 residues 504–527), CRMP-2-pee-b (GIQGEMEALRDQHGVR, rCRMP-2 residues 147–161), CRMP-3-pee-a (GIQGEMEALRDQHGVR, rCRMP-3 residues 496–519) were synthesized as described (25) and coupled to keyhole limpet hemocyanin as described (26) via additional N-terminal cysteine residues. The peptide-keyhole limpet hemocyanin conjugates were used to immunize New Zealand White rabbits (Lammers, Euskirchen, Germany). For the first immunization 200 µg of peptide dissolved in Freund’s complete adjuvant (Sigma) was injected subcutaneously. Each animal was boosted twice at intervals of 4 weeks with the same amount of antigen in incomplete Freund’s adjuvant (Sigma). For the immunization protocol, special permission according to Section 8 of the German Law on the Protection of Animals was obtained from the Bezirksregierung Köln. All rabbit antisera were used at a dilution of 1:10,000 for Western blotting. Monospecific IgG was purified by immunoaffinity chromatography with the peptides immobilized on thiol-Sepharose (Amersham Biosciences) as described (26). Purified IgG was ultrafiltered into 50 mM NaHCO₃, pH 8.5. After the addition of 10 µl of 150 mM NHS-Biotin (Pierce) the antibody solution was incubated at 4 °C overnight. IgG was then separated from the unreacted free biotin by size exclusion chromatography on NAP10 columns (Amersham Biosciences).

**Glycoosaminoglycan Affinity Chromatography—**Chondroitin sulfate was coupled to EAH-Sepharose (Amersham Biosciences) as described (18). As control columns, heparin Hitrap, SP Hitrap, and CM Hitrap 1-ml columns (Amersham Biosciences) were used. Approximately 10 mg of soluble neonatal rat brain proteins obtained after ultracentrifugation of postnuclear supernatants at 100,000 × g for 1 h were filtered (0.45 µm) and chromatographed on 1-ml analytical columns using an Äkta Explorer equipment (Amersham Biosciences). Runs on CS columns and control columns were carried out in parallel, taking advantage of the column-scouring routine of the Unicorn 3.1 software using sequential step elution with PBS containing 300 mM NaCl, 750 mM NaCl, 2 mM NaCl, and 4 mM guanidinium hydrochloride (GuaHCl) as described (18). To remove the salt from the eluent fractions and to concentrate the proteins, they were precipitated with acetone (–20 °C) and washed twice with 80% ethanol (4 °C) before Western blot analysis with the peptide-specific antibodies against CRMPs.

**Immunoprecipitation—**1-ml aliquots of soluble neonatal rat brain proteins prepared as described above were incubated with 1 µl of the different peptide-specific CRMP antisera for 1 h at 4 °C and with 10 µl of protein A-agarose (Calbiochem) for an additional hour at 4 °C with continuous rocking. The precipitates were collected by centrifugation, washed 3 times in phosphate-buffered saline, dissolved in 100 µl of Laemmli buffer, and analyzed by Western blotting with the biotinylated CRMP-Fam antibody or CS56 (Sigma) against CS.

**Results**—Before brain dissection, animals were perfused with standard mammalian Ringer’s solution, pH 7.4, followed by 3.7% formaldehyde. Brains were post-fixed for 1 h and washed extensively in tap water. After dehydration in a series of increasing ethanol concentrations, brain tissue was embedded in paraffin, and 10-µm sections were cut on a microtome (Leica RM 355 S). Sections were mounted on Histobond slides, dried for 2 days at 37 °C, and used for immunostaining. First, sections were deparaffinized and hydrated by decreasing concentrations of ethanol in H₂O. Afterward, sections were incubated in 5 % SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) for 20 min. After equilibrating in PBS, sections were treated with 1% H₂O₂ in PBS for 10 min to remove endogenous peroxidase activity. After permeabilization with 0.5% Triton X-100 in PBS for 10 min, sections were washed with PBS and incubated in a 4% bovine serum albumin, PBS, washed again, and incubated in streptavidin-peroxidase complex (ABC-kit, Vector). After a 1-h incubation, sections were washed intensively and stained with diaminobenzidine (0.05% in Tris-buffered saline). Counterstaining was done with hemalum (Mayers hemalum, 1:6 dilution in H₂O, Merck) for 5 min followed by several washes in H₂O and a final wash in tap water. Pictures were taken with a digital camera (Polaroid DMC Ie, Cambridge, UK) connected to a Zeiss Axioscope 2 (Jena). Cell Culture—Ntera-2 precursor cells (Stratagene) were grown in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal calf serum (FCS), 1-glutamine, and penicillin/streptomycin. Cell death was induced by feeding the cells with medium without FCS after repeated washings with serum-free medium. Conditioned medium was harvested after 3 days and concentrated by centrifugation at 1000 × g for 15 min to remove floating cells and debris. For Western blot analysis, conditioned medium was subjected to Q-Sepharose (Hitrap 1-ml column, Amersham Biosciences) chromatography to capture CRMPs using elution with a linear gradient from 150 mM to 1 mM NaCl in phosphate buffer.

Primary neuronal cultures were prepared as described (27) and plated onto poly-L-lysine-coated 10-cm cell culture Petri dishes (Falcon) or 8-well cell culture plates (Sarstedt). Cultures were incubated at 37 °C in humidified 10% CO₂, 90% air for 16–24 h and analyzed by phase contrast microscopy. To visualize the morphology of nuclei, cultures were fixed for 10 min in 4% paraformaldehyde, washed with PBS, and stained briefly with 4,6-diamidine-2-phenylindole (100 µg/ml in PBS). Pictures were taken with a digital camera (Axiovision, Zeiss) connected to a Zeiss Axiosvert 100M, and images were processed with the Axiovision software (Zeiss, Göttingen, Germany).

**Analytical Procedures**—Protein determination was performed with the Bradford assay or the detergent-compatible protein assay (both purchased from Bio-Rad) using bovine serum albumin as the standard. Lactate dehydrogenase activity was determined as described (28).

**RESULTS**

**Purification of CRMP-4 from Neonatal Rat Brain**—The monoclonal antibody mAb-9 used in this study recognizes a 65-kDa protein in the cell lysate fraction of neonatal rat brain (18). From this material the 65-kDa protein was captured on a Mono Q anion exchange column (Fig. 1). When steps of increasing ionic strength were applied to the column, about 30% of the cross-reacting protein was eluted with 200 mM sodium chloride, and the remaining 70% was released at 500 mM sodium chloride according to Western blot analysis (Fig. 1C). Interestingly, the electrophoretic mobility of the cross-reacting protein bands from the eluate fractions was slower in comparison to the starting material (Fig. 1C). Because the 200 mM NaCl eluate contained a lower amount of contaminating proteins than the 500 mM eluate, according to silver-stained SDS-gels (Fig. 1B), fraction 7 from the Mono Q column was subsequently fractionated using size exclusion chromatography on a Superose 12 column (Fig. 2). From this column the cross-reacting protein eluted after 11–12 ml, corresponding to a molecular mass of ~200 kDa (Fig. 2, A and C). Finally, purification to homogeneity was achieved by preparative SDS-PAGE (data not shown). In-gel digestion with trypsin yielded 22 peptides, which were analyzed by ESI-MS. In the SwissProt.r34 rat sequence database (Table I) 10/22 peptide masses fitted to a rat sequence homologous to the toxicology of CRMP-4 (29). The specificity of the CRMP-4/200 kDa (Fig. 2, A and C) massively increased when comparing to the entire mammalian data base (nrdb, EMBL Heidelberg), however, showed a more close match of 15/22 peptides to mouse CRMP-4 (mUlip, Unc33-like phosphoprotein (31)). Extension of the 19 of the 22 obtained masses could be matched exactly (data not shown). Thus, the mass spectrometric analyses indicate

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that mAb-9 recognizes collapsin response mediator protein-4 from rat brain.

Western Blot Analysis of Recombinant CRMPs with mAb-9—To confirm that mAb-9 recognizes CRMP-4 and to determine the specificity of the antibody, the coding regions of rat CRMP-1–4 sequences were amplified by PCR, sequenced, and expressed as N-terminally His-tagged fusion proteins in E. coli. All four CRMPs were found in inclusion bodies. When equal amounts of the four recombinant proteins were analyzed by Western blotting, all recombinant proteins were detected with an anti-polyhistidine antibody (Fig. 3B). Recombinant CRMP-4 clearly reacted with mAb-9, whereas CRMP-2 was not detected even after prolonged exposure of radiography films (Fig 3A). Moreover, CRMP-1 and -3 cross-reacted weakly with mAb-9 (Fig. 3A). In summary, mass spectroscopic experiments and the immunological analysis of recombinant CRMPs consistently demonstrated that mAb-9 recognizes an epitope that is present on CRMP-4.

Characterization of Peptide-specific Antibodies against CRMPs—To distinguish different members of the CRMP family, we produced peptide-specific polyclonal antisera. Peptides were designed from regions of the CRMP amino acid sequence that displayed marked divergence between the CRMPs. Furthermore, a sequence was selected that is conserved among the CRMP family members to generate a pan-specific antibody. After immunization of rabbits, five different antibodies were obtained termed anti-CRMP-1 to -4 and anti-CRMP-Fam. Western blots of the bacterially expressed recombinant CRMPs demonstrated binding of anti-CRMP-Fam to all four recombinant CRMPs (Fig. 3C) and mono-specific binding of the antibodies anti-CRMP-1, -2, and -4 to the corresponding recombinant proteins (Fig. 4, A, B, and D). Anti-CRMP-3 strongly bound to recombinant CRMP-3 and showed weak cross-reactions with CRMP-1 and -2 (Fig. 4C).

Interaction of CRMPs with Glycosaminoglycans—The mAb-9 was raised against chondroitin sulfate-binding proteins that were solubilized with CHAPS from the particulate fraction of neonatal rat brain. This raises the possibility that CRMPs could be cytosolic associates of membrane-associated protein complexes that contain receptors for ECM proteoglycans. In this case, soluble CRMPs might not necessarily bind to chondroitin sulfate. Thus, we examined if CRMPs from the soluble fraction of neonatal rat brain bind to chondroitin sulfate-Sepharose columns. First, eluates from a chondroitin-6-sulfate-Sepharose column were screened for the presence of CRMPs using the peptide-specific antibodies. All four CRMPs bound to the column (Fig. 5). Although substantial amounts of the bound CRMPs were released on washing of the column at moderate stringency (Fig 5, lane 1 of each panel), limited quantities of each CRMP remained on the column even after harsh washing with 2 M NaCl and were only eluted by the chaotropic salt GuaHCl (Fig. 5, lane 4 of each panel). Almost identical elution profiles of CRMPs were observed with a chondroitin-4-sulfate column (data not shown). As the control we performed parallel chromatographies on a heparin-Sepharose column under exactly the same experimental conditions. Although all four CRMPs bound quantitatively to the heparin column, they were eluted completely by washing the column with 300 mM NaCl (Fig. 6, lane 1 of each panel), none of the CRMPs was eluted with GuaHCl, as seen for both chondroitin sulfate columns (Fig. 6, lane 4 of each panel). Taken together, these chromatography profiles indicate a weak charge-mediated interaction of CRMPs with heparin but tight binding of sub-
classes of each CRMP to chondroitin sulfates.

To rule out the possibility that soluble CRMPs were retained on chondroitin sulfate columns because they interact with putative contaminants of these glycosaminoglycans from their biological sources (i.e. shark cartilage for C-6-S and bovine trachea for C-4-S), we carried out immuno-co-precipitation experiments as an independent approach. Anti-CRMP4 and anti-CRMP-Fam precipitated detectable amounts of CRMPs (Fig. 7A). Western blot analysis with the monoclonal antibody CS56 against chondroitin sulfate demonstrated co-precipitation of characteristic smears proteoglycan bands after precipitation of soluble brain-derived proteins with anti-CRMP4 and the anti-CRMP-Fam but not after incubation with a preimmune serum (Fig. 7B). Thus, immuno-co-precipitation data confirmed that soluble CRMPs bind to chondroitin sulfate.

Localization of CRMP-4 and Chondroitin Sulfate in Overlap-
ping Regions of the Cerebral Cortex—To determine sites in brain tissue where an interaction between CRMPs and chondroitin sulfate may take place, we examined the immunohistochemical distribution of CRMP-4 and chondroitin sulfate in the cerebral cortex of neonatal rat brain. CRMP-4-positive cells were present in the upper part of the cortical plate. Interestingly, within these cells nuclei were strongly stained (Fig. 8, A and C). Moreover, there was a fine reticular CRMP-4 staining without obvious relationship to cellular structures in the marginal zone and a diffuse labeling of the subplate and prospective white matter (Fig. 8, A and C). Chondroitin sulfate, on the other hand, was expressed as a reticular meshwork in the marginal zone (Fig. 8, B and D). Furthermore, the subplate was diffusely stained. In summary, CRMP-4 and chondroitin sulfate were partly expressed in the same regions of the neocortex.

In agreement with published data on the naturally occurring cell death in the developing rodent brain (32–34), we found numerous pyknotic nuclei in the upper cortical plate, immediately beneath the marginal zone corresponding to layer II of the mature neocortex (Fig. 8, D, arrowheads).

Release of CRMPs to the Extracellular Space—The biochemical interaction of CRMPs with chondroitin sulfate in vitro obviously raises the question of under what conditions this segregation might break down and in what compartment the interaction could become relevant in vivo. The almost congruent reticular staining patterns of CRMP-4 and CS in the marginal zone of the cerebral cortex suggested that CRMPs may be released to the extracellular space, e.g. after the programmed death of neurons. To substantiate that CRMPs are released to the extracellular space, we screened cell culture supernatants of neural cells for the presence of CRMPs. First, in serum-free conditioned media of NTera-2 precursor cell cultures we detected CRMP immunoreactivity after capture on a Q-Sepharose column (Fig. 9A). The fact that no CRMP-like molecules were detected in fetal calf serum (Fig. 9B) rules out the possibility that residual traces of FCS are the source of CRMP-like immunoreactivity in these supernatants. Because serum-starved cultures contained many dead cells on microscopic examination and considerable activity of the cytosolic marker enzyme lactate dehydrogenase (LDH) (Fig. 9C), we assume that CRMP-like molecules possibly were released from the cytosol of NTera-2 precursor cells that underwent cell death. Our data do
not exclude the possibility, however, that active mechanisms of release may exist. Second, primary cultures of neocortical neurons were studied for the release of CRMPs. These cultures had been characterized previously and contained >95% microtubule-associated protein 2-positive neurons (11, 18, 27). When these neuron cultures were grown in the presence of HEK293 cell-conditioned medium to provide trophic support, no lactate dehydrogenase activity was detected in the supernatants of these cultures. However, we observed that ~50% of cells underwent cell death with compacted rounded cell morphology (Fig. 10, A and B) and pyknosis of the nuclei (Fig. 10, C, D, and E) independent on the initial plating density. Supernatants of these cultures contained CRMPs and their amount paralleled the extent of cell death (Fig. 10F). Almost all of the released CRMP was CRMP-4 (Fig. 10G) since we were not able to detect significant amounts of the other CRMPs in the supernatant even after prolonged film exposure (data not shown). Taken together, these experiments indicated that NTera-2 precursor cells and neocortical neurons release CRMPs to the extracellular compartment and that naturally occurring cell death may be a possible release mechanism.

**DISCUSSION**

Previously, we generated the monoclonal antibody mAb-9 against chondroitin sulfate-binding proteins from neonatal rat brain. Soluble proteins (10 mg) from neonatal rat brain were loaded on a heparin-Sepharose column, washed with 300 mM NaCl (1), and eluted with 750 mM NaCl (2), 2 M NaCl (3), or 4 M GuHCl (4). All fractions were tested in Western blot with the different monospecific CRMP antisera as indicated on the right. L, load; F, flow-through. Bars on the left indicate the position of apparent molecular weight (MW) marker bands (in kDa).

**Fig. 6. Heparin affinity chromatography of soluble CRMPs from rat brain.** Soluble proteins (10 mg) from neonatal rat brain were precipitated with the peptide-specific anti-CRMP antisera as indicated above the lanes or preimmune serum (P) and analyzed by Western blotting with the biotinylated pan-specific anti-CRMP-Fam antibody (A) or the chondroitin sulfate-specific CS56 antibody (B). Positions of apparent molecular weight marker bands are indicated on the left. (B, control lane with soluble brain proteins).

**Fig. 7. Immuno-co-precipitation of CRMPs and chondroitin sulfate.** Soluble proteins from neonatal rat brain were precipitated with the peptide-specific anti-CRMP antisera as indicated above the lanes or preimmune serum (P) and analyzed by Western blotting with the biotinylated pan-specific anti-CRMP-Fam antibody (A) or the chondroitin sulfate-specific CS56 antibody (B). Positions of apparent molecular weight marker bands are indicated on the left. (B, control lane with soluble brain proteins).

**Fig. 8. Immunohistochemistry of chondroitin sulfate and CRMP-4 in the neocortex of newborn rat.** A, low power micrograph of the cerebral cortex after staining with anti-CRMP-4 (brown). Nuclei were counterstained with hemalum (blue). Bar, 50 μm. B, staining with CS56. C, high power micrograph of the pia mater, marginal zone, and upper cortical plate (later layers II/III) stained with anti CRMP-4. Bar, 20 μm. D, section corresponding to C stained with CS56. Arrowheads, pyknotic nuclei.
This antibody recognizes a 65-kDa protein with laminar expression in the cerebral cortex (18). In the present study, we identified this protein as collapsin response mediator protein-4 (CRMP-4/Ulip (31, 35)) based on mass spectrometric analysis of the purified protein and Western blot analysis of recombinant CRMP-4. Furthermore, we obtained evidence that CRMP1, -2, -3, and -4 interact with chondroitin sulfate proteoglycans. The collapsin response mediator proteins form a family of five homologues, the first of which (formerly called CRMP-62, now termed CRMP-2) was identified by expression cloning as a signal transduction molecule, mediating the growth cone collapse activity of semaphorin 3A/collapsin on peripheral sensory neurons (30). The rat orthologue (called TOAD-64) was identified as a marker of differentiating post-mitotic neurons re-expressed after nerve lesions in the adult animal (29). CRMP-4 (mUlip) (31) was cloned in mouse as a phosphoprotein cross-reacting with an anti-stathmin antibody and was later identified in rat and human by homology screening (35, 36). Recently, CRAM/CRMP-5 was cloned as a protein that interacts with CRMP-3 (37), with a glycine transporter (38), and that cross-reacts with an anti-ZAP-70 antibody (39). CRMPs share sequence similarities with dihydropyrimidinase and with the gene product of the unc-33 gene of *Caenorhabditis elegans*, which is involved in axonal pathfinding (30).

Different phosphorylation states of CRMPs exist (31, 40) and may account for slightly different electrophoretic mobilities of the protein bands recognized by mAb-9 (Fig. 1C), since in the absence of phosphatase inhibitors like orthovanadate, the protein may undergo dephosphorylation, which may markedly influence the apparent molecular weight as determined by SDS-PAGE (41). Moreover, differences in phosphorylation may explain why CRMP immunoreactivity eluted from the Mono Q
column in two distinct peaks (Fig. 1C). On the other hand, taking into account that mAb-9 weakly cross-reacts with CRMP-1 and CRMP-3 (Fig. 3A), the second peak could also represent a different CRMP. Interestingly, on size exclusion chromatography CRMP-4 migrated with a velocity corresponding to a molecular size of ~200 kDa (Fig. 2, A and C). This obvious discrepancy to the apparent $M_d$ of 65 kDa, as determined by SDS-PAGE (Figs. 1C and 2C), could reflect the tendency of CRMPs to form heterotetramers under native conditions (42).

In addition to their heterotetramerization, CRMPs interact with several other proteins. In sensory neurons, CRMP-2 is phosphorylated on Thr-555 by Rho kinase upon stimulation of growth cone collapse by lysophosphatidic acid (43). Although this lysophosphatidic acid-dependent signaling pathway does not depend on semaphorin 3A, activity of phospholipase D2 is inhibited by CRMP-2 was found to be regulated by semaphorin 3A in PC12 cells (44). Furthermore, semaphorin 3A enhances tyrosine phosphorylation of CRMP-2 and CRMP-5 via Pes/Fps tyrosine kinase (45). Recently, it was shown that CRMP-2 binds to tubulin heterodimers and promotes microtubule assembly (46). Other interactions of CRMPs probably exist since CRMP-2 copurifies with dichlorophenol-indophenol oxidoreductase, aldolase C, and glyceraldehyde-3-phosphate dehydrogenase from adult bovine brain, suggesting complex formation of these proteins (47). All interactions of CRMPs mentioned so far involve proteins that are exposed to the cytosol. In this study, however, we show that CRMPs interact with chondroitin sulfates. Chondroitin sulfates represent an entirely novel category of interaction partners for CRMPs, since they are carbohydrates, and they are assumed to reside mainly in the extracellular space. Similar to heparin, chondroitin sulfates are polyanionic glycosaminoglycans, but they carry a lower density of negative charges.

Interestingly, the elution patterns of CRMPs from heparin and chondroitin sulfate columns differed markedly. Although soluble CRMPs bound completely to a heparin column (Fig. 6) and were eluted quantitatively at moderate ionic strength (300 mM NaCl), indicating a charge-mediated interaction of low affinity, only about 50% of each CRMP bound to chondroitin sulfate columns (Fig. 5). This incomplete binding to CS columns could reflect competition of the abundant endogenous brain-derived CS proteoglycans with the immobilized glycosaminoglycan, as suggested by our immuno-coprecipitation experiments (Fig. 7B). Importantly, the CRMPs were not completely recovered from the CS columns even after stringent washing with buffer containing 2 mM NaCl, as evidenced by the presence of CRMPs in eluates obtained with buffer containing the chaotropic salt guanidinium hydrochloride (Fig. 5, lane 4 in each panel). Thus, certain CRMP forms obviously engage in high affinity interactions with chondroitin sulfates that were not observed on heparin and which cannot be attributed solely to ion exchange effects.

Chondroitin sulfates are abundant in the extracellular matrix of the developing brain (1), whereas CRMPs as proteins without secretory leader peptides are assumed to exist well separated in the cytosol (30). The immunohistochemical fine reticular or diffuse staining patterns of CS in the marginal zone and subplate of the cerebral cortex (Fig. 8, B and D) are in agreement with published data (9, 48, 11) and presumably represent extracellular localizations of the glycosaminoglycan. No cytoplasmic CS was detected in the present study, and in the literature, evidence for cytoplasmic proteoglycans in the CNS is limited to the adult stage (49). On the other hand, CRMP-4 was found (i) in cells of the cortical plate mainly in nuclei, (ii) in the marginal zone with a reticular staining pattern very similar to the CS-staining, and (iii) in the subplate and prospective white matter (Fig. 8, A and C). The nuclear localization of CRMP-4 is consistent with reports on the targeting of a G protein-CRMP-1 fusion protein to nuclei in lung cancer cells (50) and of CRMP-2-positive nuclear inclusions after overexpression in Neuro2A cells (51). The similar reticular staining pattern of CS (Fig. 8, B and D) and CRMP-4 (Fig. 8, A and C) in the marginal zone, however, suggest that CRMP-4 may be present in extracellular compartment of the cerebral cortex. Taking into account that some proteins lacking a secretory leader peptide are targeted extracellularly (e.g. bovine fibroblast growth factor in MG-63 cells (52)), release of CRMPs to the extracellular space may occur under particular circumstances. Obviously, it is difficult to prove rigorously that a protein is located in the extracellular space based on immunohistochemical analysis of brain tissue. An important argument for the existence of extracellular CRMPs is the presence of these proteins in cell culture supernatants of NTera-2 precursor cells (Fig. 9A) and of neocortical neurons (Fig. 10, F and G). CRMPs were detected in the extracellular compartment, concomitant with cell death, as judged from the release of the cytosolic marker lactate dehydrogenase in the NTera-2 precursor cultures (Fig. 9C) and the presence of condensed, pyknotic chromatin (Fig. 10, C, D, and E) in ~50% of the primary neocortical neurons, indicating naturally occurring (programmed) cell death. Naturally occurring cell death is a widely distributed phenomenon during the development of the central nervous system (33) and is found in the perinatal rat neocortex mainly in the future layers II/III (32, 34). In these layers we found particularly strong cellular CRMP-4 staining (Fig. 8, A and C) and numerous pyknotic nuclei (Fig. 8D, arrowheads). Moreover, the non-cellular reticular CRMP-4 immunoreactivity in the marginal zone toward the pial surface and in the cortical plate is consistent with the formation of diffusion gradients of released CRMP-4 away from the zone of neuron death. Binding to CS proteoglycans may help to shape and stabilize gradients of diffusible CRMPs (53). On the other hand, CS could participate in the control of CRMP release from dying neurons since it inhibits the death of neocortical neurons in vitro (11). Thus, CS could be an important regulator of the release and distribution of extracellular CRMPs in the cerebral cortex. On the other hand, CRMP-3 and semaphorin 3A could regulate the release of CRMPs from dying cells since it has been shown to promote the apoptosis of certain neuron classes (54).

The function of extracellular CRMPs could relate to the establishment of contacts with afferents, since the spatiotemporal pattern of naturally occurring cell death in the neocortex correlates with the arrival and settlement of cortical afferents at the different cortical levels (32). Interestingly, Emerling and Lander (17) obtained evidence that CS-bound soluble cues dramatically influence the growth of thalamic neurites within the cerebral cortex. Thus, it is tempting to speculate that CRMPs may belong to these CS-bound cues. However, screening for functional effects of purified CRMP applied in cell culture paradigms and careful analysis of CRMP transgenes will help to clarify the as yet unknown physiological roles of extracellular CRMPs in the future.

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