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The Unc-33-like phosphoprotein/collapsin response mediator protein (Ulip/CRMP) family consists of four homologous phosphoproteins considered crucial for brain development. Autoantibodies produced against member(s) of this family by patients with paraneoplastic neurological diseases have made it possible to clone a fifth human Ulip/CRMP and characterize its cellular and anatomical distribution in developing brain. This protein, referred to as Ulip6/CRMP5, is highly expressed during rat brain development in postmitotic neural precursors and in the fasciculi of fibers, suggesting its involvement in neuronal migration/differentiation and axonal growth. In the adult, Ulip6/CRMP5 is still expressed in some neurons, namely in areas that retain neurogenesis and in oligodendrocytes in the midbrain, hindbrain, and spinal cord. Ulip2/CRMP2 and Ulip6/CRMP5 are coexpressed in postmitotic neural precursors at certain times during development and in oligodendrocytes in the adult. Because Ulip2/CRMP2 has been reported to mediate semaphorin-3A (Sema3A) signal in developing neurons, in studies to understand the function of Ulip6/CRMP5 and Ulip2/CRMP2 in the adult, purified adult rat brain oligodendrocytes were cultured in a Sema3A-conditioned medium. Oligodendrocytes were found to have Sema3A binding sites and to express neuropilin-1, the major Sema3A receptor component. In the presence of Sema3A, these oligodendrocytes displayed a dramatic reduction in process extension, which was reversed by removal of Sema3A and prevented by anti-neuropilin-1, anti-Ulip6/CRMP5, anti-Ulip2/CRMP2 antibodies, or VEGF-165, another neuropilin-1 ligand. These results indicate the existence in the adult brain of a Sema3A signaling pathway that modulates oligodendrocyte process extension mediated by neuropilin-1, Ulip6/CRMP5, and Ulip2/CRMP2, and they open new fields of investigation of neuron/oligodendrocyte interactions in the normal and pathological brain.

Key words: Ulip/CRMP; oligodendrocyte; Sema3A; process extension; anatomical expression; neurodegenerative disorders

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adult rat brain. Because we observed an expression in adult oligodendrocytes as described for Ulip6/CRMP2 (Ricard et al., 2000), we compared the distribution of these two proteins and found that they were coexpressed at certain times during development and in oligodendrocytes. In studies to understand the function of Ulip6/CRMP5 and Ulip2/CRMP2 in adult, purified adult rat brain oligodendrocytes were submitted to Sema3A, a semaphorin known for its attractive/repulsive properties on growing axons (Püschel, 1999; Bagnard et al., 1998, 2000). These oligodendrocytes were found to have Sema3A binding sites and to express neuropilin-1, the major component of the Sema3A receptor complex (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). In the presence of Sema3A, the oligodendrocyte process extensions displayed a dramatic decrease that was reversed by removing the Sema3A or prevented by anti-neuropilin-1, anti-Ulip6/CRMP5, or anti-Ulip2/CRMP2 antibodies or VEGF-165, another ligand for neuropilin-1 (Miao et al., 1999). These results indicate the existence of a Sema3A signaling pathway controlling oligodendrocyte process extension in adult brain via neuropilin-1, Ulip6/CRMP5, or Ulip2/CRMP2.

MATERIALS AND METHODS

Reagents. Unless specified otherwise, all reagents were purchased from Sigma (L’Isle d’Abeau, France).

Production of recombinant proteins. cDNAs coding for mouse Ulip1/CRMP4 (GenBank accession number X87817), Ulip2/CRMP2 (GenBank accession number Y10339), Ulip3/CRMP1 (GenBank accession number Y90080), and Ulip4/CRMP3 (GenBank accession number Y90079), kindly provided by A. Sobel (Institut National de la Sante et de la Recherche Médicale, Paris, France), were cloned in-frame with a flag sequence (Sigma) in the pSG5 vector (Stratagene, Amsterdam, The Netherlands) and used to produce recombinant proteins in HeLa cells as described previously (Ricard et al., 2000). Human Ulip6/CRMP5 cDNA (GenBank accession number AF264015), cloned in-frame with the Lac-Z gene in pBluescript KS, was used to produce bacterial recombinant protein. Briefly, Escherichia coli cells were grown for 1 hr at 37°C, then Ulip6/CRMP5 expression was induced with isopropyl-1-thio-β-d-galactopyranoside (0.1 mM). After 3 hr at 37°C, the cells were lysed by sonication, and the soluble extract containing the Ulip6/CRMP5 recombinant protein was obtained by centrifugation for 10 min at 2000 × g.

Antibodies. The antibodies used to generate specific antibodies were KEMTGPLADTPRTPVTRHGG (amino acids 505–524) for anti-Ulip6/CRMP5, LEDGTLHVTEGS and ITPEGHVLSPREEPE (amino acids 454–465 and 217–232, respectively) for anti-Ulip2/CRMP2, LITSFEKWEAADTKS (amino acids 117–131) for anti-Ulip3/CRMP1, and EHDSHAQLRWRVL (amino acids 664–676) for anti-neuropilin-1. Synthetic peptides were conjugated to keyhole limpet hemocyanin and used to immunize rabbits or rats as described previously (Honnorat et al., 1999). The antibodies were purified from anti-Ulip6/CRMP5, anti-Ulip3/CRMP1, and anti-neuropilin-1 antisera using the corresponding immobilized peptide.

Protein samples. Male rats (要素, Iffa-Credo, L’Arbresle, France) were anesthetized with pentobarbital and perfused in situ with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, then the brains were fixed by immersion for 12 hr in 4% paraformaldehyde in PB. After three rinses and overnight incubation in PB/20% sucrose, the brains were frozen at −60°C in methyl-butane. Embryos [embryonic day 16 (E16) and E19, respectively] were removed from the anesthetized pregnant female rats and fixed by immersion for 1 hr in 4% paraformaldehyde, then treated in the same way as the adult tissues. Sagittal cryostat sections (14 μm thick) were collected on Superfract Plus slides (Polylabo, Strasbourg, France) and stored at −20°C until required.

Immunohistochemistry. Four adult male, four 2-week-old [postnatal day 15 (P15)], four 5-d-old (P5), and four pregnant female rats (要素, Iffa-Credo) were used. The adult male and P15 rats were anesthetized with pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, then the brains were removed and post-fixed in 4% paraformaldehyde for 12 hr. The brains of anesthetized P5 rats were fixed by immersion for 12 hr in 4% paraformaldehyde in PB. After three rinses and overnight incubation in PB/20% sucrose, the brains were frozen at −60°C in methyl-butane. Embryos [embryonic day 16 (E16) and E19, respectively] were removed from the anesthetized pregnant female rats and fixed by immersion for 1 hr in 4% paraformaldehyde, then treated in the same way as the adult tissues. Sagittal cryostat sections (14 μm thick) were collected on Superfrac Plus slides (Polylabo, Strasbourg, France) and stored at −20°C until required.

Immunohistochemistry was performed as described previously (Honnorat et al., 1998). Briefly, tissue sections were conjugated to keyhole limpet hemocyanin and used to immunize rabbits or rats as described previously (Honnorat et al., 1999). The antibodies were purified from anti-Ulip6/CRMP5, anti-Ulip3/CRMP1, and anti-neuropilin-1 antisera using the corresponding immobilized peptide.

Purified oligodendrocyte cultures. Oligodendrocytes were isolated from six 4-week-old Sprague Dawley male rats (要素, Iffa-Credo) using the procedure of Lisak et al. (1981), as modified by Lubetzkii et al. (1988). Freshly isolated cells were plated on poly-L-lysine-coated glass coverslips (OSL, Maurepas, France) in 24-well plates (Costar Corporation, Cambridge, MA) at a density of 10^5 cells per well, initially for 1 hr in DMEM (Life Technologies, Cergy-Pontoise, France) containing 10% fetal calf serum (FCS; Eurobio, Les Ulis, France) to facilitate attachment, and then in standard culture medium consisting of Bottenstein and Sato medium (Bottenstein and Sato, 1979) supplemented with 5 U/ml of penicillin and 5 μg/ml of streptomycin (Life Technologies).
Sem3A recombinant protein in HBSS supplemented with 20% FCS, washed three times in PBS, then fixed for 1 hr in 4% paraformaldehyde. After one wash in PBS, endogenous phosphatases were heat-inactivated at 65°C for 50 min, then the preparations were equilibrated for 20 min with AP buffer (100 mM Tris, 100 mM NaCl, and 5 mM MgCl2, pH 9.5), and the bound AP-Sem3A was visualized using a staining solution containing 34 mg/ml of Nitro-blue-tetrazolium and 18 mg/ml of 5-bromo-4-chloro-3-indolylphosphate (Roche) in AP buffer. Immunostaining with a monoclonal anti-Rip antibody, an oligodendrocyte marker (Friedman et al., 1989), was then used to visualize oligodendrocytes. The controls consisted of performed experiments with oligodendrocytes incubated in culture medium without recombinant protein or in the presence of an excess of untagged Sem3A.

Oligodendroocyte process extension assay. Highly purified mature oligodendrocytes were obtained and grown for 48 hr in BS medium (see above); then the BS medium was replaced with either Sem3A-conditioned medium (Sem3A medium) or obtained from human embryonic kidney (HEK 293) cells transfected with Sem3A expression vector, as described previously (Bagnard et al., 1998), or control medium from untransfected HEK 293 cells. Purified oligodendrocytes were also incubated for 48 hr in Sem3A medium containing either 50 ng/ml of VEGF (Miao et al., 1999) or various concentrations of antibodies (2, 4, or 8 µg/ml of immunopurified anti-neuropilin-1, anti-Ulip6/CRMP5, or anti-Ulip3/CRMP1 antibodies or 4, 8, or 20 µg/ml of IgG purified from anti-Ulip2/CRMP2 antisera and preimmune sera). Incubation with the different anti-Ulip/CRMP antibodies at 8°C (see below), as described for other neural cells (Fishman et al., 1990, 1991; Greenlee et al., 1993). The cultures were then fixed in 4% paraformaldehyde and analyzed. They were first immunostained using the Rip monoclonal antibody and microphotographed using a 40× objective (Zeiss). Processes were quantified on the photographs using a grid composed of concentric circles separated by 10 µm and centered on the cell body (see Fig. 9). The number of intersections between the circles and processes was counted for each cell, defining a branching index (BI); 20 cells were counted in each test sample to determine the mean BI. The results were confirmed in at least two independent experiments. Effects of treatments were quantified using the percentage extension compared with that under control conditions calculated as [(BI in control medium − BI in Sem3A medium)/BI in control medium] × 100. The statistical significance of the results was evaluated using the unpaired Student’s t-test.

Oligodendrocyte viability assay. Viability of the oligodendrocytes cultured for 48 hr with or without Sem3A was estimated by propidium iodide and trypan blue staining. Cells adhering on the glass coverslides or recovered in the culture medium were quantified.

**Antibody penetration in living oligodendrocytes.** Highly purified oligodendrocytes, grown for 48 hr in BS medium, were incubated for 1 hr with rabbit anti-Ulip2/CRMP2 or anti-Ulip6/CRMP5 IgG (30 µg/ml) either at 37°C or at 4°C for control. Cells were then washed twice in PBS and fixed for 20 min in 4% paraformaldehyde. After two washes in PBS, cells were incubated for 10 min in PBS containing 0.2% gelatin and 0.1% Triton X-100 and then with Alexa 488-conjugated rabbit anti-IgG (Molecular Probes, Interchim, Montluc, France) for 45 min. Rabbit IgGs were clearly detected in the cytoplasm of 70% of the oligodendrocytes when antibody incubation was performed at 37°C (see Fig. 10E). No labeling was observed when antibodies were incubated at 4°C (see Fig. 10G), indicating that IgG penetration in living oligodendrocytes is a physiologic mechanism.

All animal experiments were performed in accordance with French legal requirements (decree 87–848) and with the European Community Council Directive of November 24, 1986 (86/609/EEC).

**RESULTS**

**Molecular characterization and tissue distribution of human Ulip6/CRMP5**

A human spinal cord cDNA library was screened using an anti-CV2 serum from a patient with PND and small-cell lung carcinoma that recognized a 66 kDa protein on Western blots of newborn rat brain protein extracts, but did not recognize any of the four previously known Ulip/CRMP recombinant proteins. This led to the identification of one partial-length clone (C97) containing a 1.6 kb cDNA insert yielding a 90 amino acid open reading frame that showed 35% homology with the C-terminal region of the four known human Ulip/CRMP proteins. The cDNA containing the full-length coding region was obtained by screening the same library with a radioactive probe corresponding to the coding region of C97 (270 bp). A 2 kb cDNA, referred to as Ulip6/CRMP5, which contains an open reading frame coding for 564 amino acids, was isolated. The C-terminal region of this protein was identical to the 90 amino acids encoded by C97. On Western blots, the Ulip6/CRMP5 recombinant protein was recognized by all 20 anti-CV2 sera tested (Fig. 1C) but not by 100 sera from patients without PND (half of them having small-cell lung carcinoma), suggesting that Ulip6/CRMP5 was the major protein that recognized a 66 kDa protein on Western blots of newborn rat brain protein extracts, but did not recognize any of the four previously known Ulip/CRMP recombinant proteins. This led to the identification of one partial-length clone (C97) containing a 1.6 kb cDNA insert yielding a 90 amino acid open reading frame that showed 35% homology with the C-terminal region of the four known human Ulip/CRMP proteins. The cDNA containing the full-length coding region was obtained by screening the same library with a radioactive probe corresponding to the coding region of C97 (270 bp). A 2 kb cDNA, referred to as Ulip6/CRMP5, which contains an open reading frame coding for 564 amino acids, was isolated. The C-terminal region of this protein was identical to the 90 amino acids encoded by C97. On Western blots, the Ulip6/CRMP5 recombinant protein was recognized by all 20 anti-CV2 sera tested (Fig. 1C) but not by 100 sera from patients without PND (half of them having small-cell lung carcinoma), suggesting that Ulip6/CRMP5 was the major
antigen recognized by anti-CV2 antibodies. The overall sequence of the Ulip6/CRMP5 cDNA (GenBank accession number AF264015) consists of 3074 bp made up of a 162 bp 5’-noncoding region, a 1692 bp protein coding region, and a 1220 bp 3’-noncoding region. The initiation codon was assigned to the Met codon at position 163–165. The deduced protein sequence predicted a protein with a molecular mass of 61.424 kDa and an isoelectric point of 7.46.

Alignment of the sequence of the Ulip6/CRMP5 protein with those for the four known human Ulip/CRMP proteins showed 48–50% identity. Ulip6/CRMP5 and the other members of the family share the same degree of identity (~33%) with the Caenorhabditis elegans gene product, unc-33 (Byk et al., 1998), a gene required for neurite outgrowth and axonal guidance (Li et al., 1992). The Ulip6/CRMP5 sequence contains consensus sites for several protein kinases, such as casein kinase II (eight sites), tyrosine kinase (two sites), protein kinase A (one site), and protein kinase C (eight sites). Alignment of the sequence of the human Ulip6/CRMP5 protein with those of rat CRM (GenBank accession number AB029432) and mouse CRMP5 (GenBank accession number AF249295) showed 97% identity, and comparison of the cDNA sequences showed >80% identity.

Northern blot analysis using a Ulip6/CRMP5 RNA probe identified a 5.5 kb band in human brain mRNA, whereas mRNAs prepared from various adult human peripheral tissues gave no hybridization signal (Fig. 1A), indicating preferential expression of Ulip6/CRMP5 mRNA in neural tissue. Expression of Ulip6/CRMP5 protein was analyzed by Western blotting using a rabbit polyclonal antiserum that, as shown in Figure 1D, recognized the Ulip6/CRMP5 recombinant protein but not the other four Ulip/CRMPs. As for the other Ulip/CRMPs (Hamajima et al., 1996; Wang and Strittmatter, 1996; Byk et al., 1998), Ulip6/CRMP5 protein was highly expressed in the embryonic brain and showed a dramatic downregulation during ontogenesis, as illustrated in the cerebellum (Fig. 1B). During development, Ulip6/CRMP5 was detected mainly in brain and lightly in muscle (Fig. 1B, P1). In adult rat tissue extracts, expression of Ulip6/CRMP5 was seen in brain and, at a lower level, in testis but not in muscle (Fig. 1B).

**Distribution of Ulip6/CRMP5 in the developing and adult rat brain**

To investigate the function of Ulip6/CRMP5, we determined the distribution pattern of the mRNA and protein using in situ hybridization or immunohistochemistry, respectively, on sections of E16 and E19 rat embryo and postnatal rat brain (P5, P15, and adult). Sense probes and preimmune serum, used as controls, gave no signal (data not shown). Ulip6/CRMP5 mRNA and protein were found to be highly expressed in the embryonic (E16 and E19) and postnatal (P5 and P15) brain and downregulated in the adult. The distribution of the protein was studied using anti-Ulip6/CRMP5 antibodies, which specifically recognized recombinant Ulip6/CRMP5 protein (Fig. 1D). The results are summarized in Table 1 and described in detail below. The observed distribution was identical to that described previously with anti-CV2 sera (Honnorat et al., 1996, 1998, 1999). In addition, the distribution of Ulip6/CRMP5 mRNA and protein in the adult brain was similar to that described for Ulip2/CRMP2 (Ricard et al., 2000), so Ulip6/CRMP5 and Ulip2/CRMP2 expression patterns were compared in detail in embryonic and postnatal rat brain.

### Table 1. Immunohistochemical study of Ulip6/CRMP5 protein expression

<table>
<thead>
<tr>
<th>Location</th>
<th>E16</th>
<th>P5</th>
</tr>
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<tbody>
<tr>
<td>Retina</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thalamus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Midbrain</td>
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<td>+</td>
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<tr>
<td>Cerebellum</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pons</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Dorsal root ganglia</td>
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**Distribution of Ulip6/CRMP5 mRNA and protein in the developing brain**

In the embryo and during the first postnatal days (P5), immunolabeling and in situ hybridization gave globally similar results.
Expression of Ulip6/CRMP5 mRNA and protein in cells expressing mRNAs. All ventricular regions, such as in the cortex (Fig. 2), indicating expression of Ulip6/CRMP5 protein in cells expressing mRNAs. All ventricular regions, such as in the cortex (Fig. 2), expressing mRNAs. All ventricular regions, such as in the cortex (Fig. 2), indicating expression of Ulip6/CRMP5 protein in cells

Distribution of Ulip6/CRMP5 mRNA and protein in the adult brain

Between P20 and the adult, the pattern of expression of Ulip6/CRMP5 was constant. In the adult brain, neurons expressing Ulip6/CRMP5 were identified by their anatomical localization, size, and shape. Ulip6/CRMP5 mRNA and protein were expressed in migrating neurons in the rostral migratory stream of the olfactory bulb, scarce neurons throughout the neocortex (Fig. 5A,B), and granular neurons in the juxta-hilar portion of the granular cell layer of the hippocampus (Fig. 5C,D). Moreover, low expression of Ulip6/CRMP5 mRNA in the absence of detectable protein was seen in a few neurons, namely the molecular and granular neurons of the IGL and a few Purkinje cells in the cerebellum (Figs. 3C, 4E). Similarly, Ulip2/CRMP2 mRNA was expressed in Purkinje cells and to a lesser extent in molecular and granular neurons of the IGL (Fig. 5F), despite the absence of detectable Ulip2/CRMP2 protein in these neurons (Fig. 4F). The presence of Ulip6/CRMP5 and/or Ulip2/CRMP2 mRNAs in some neurons in the absence of detectable protein indicates either rapid turnover of the protein or translational or post-translational regulation of the protein. Phosphorylation, glycosylation, or association of Ulip6/CRMP5 and Ulip2/CRMP2 with other proteins (Bulliard et al., 1997; Wang and Strittmatter, 1997; Inatome et al., 2000) could limit the recognition of the protein by the antibodies.

In the adult brain, the strongest Ulip6/CRMP5 mRNA and protein expression was seen in oligodendrocytes of the myelinated tracts of the spinal cord, hindbrain, midbrain, and cerebellum (Figs. 3C, 4E, 5F). Ulip6/CRMP5 mRNA and protein were detected in small cells distributed in rows in the myelinated tracts and double labeled with the oligodendrocyte-specific Rip monoclonal antibody (data not shown), as described previously using anti-CV2 sera (Honnorat et al., 1996, 1998). Ulip6/CRMP5-expressing oligodendrocytes were detected according to an increasing rostral to caudal gradient, starting in the anterior part of the basal cerebral peduncle. In the brainstem, the highest number of Ulip6/CRMP5-positive oligodendrocytes was found in the cerebellar peduncles (Fig. 6A), the spinal tract of the trigeminal nerve, the tractus pyramidalis, and the ventrospino-cerebellar tract. Within the nerve tracts, immunostained cells were widespread and bore thin stained processes clinging to the myelin sheath (Figs. 5F, 6A). The spinal cord contained the greatest number of immunostained cells (Fig. 5F). All along the spinal cord, many Ulip6/CRMP5-positive oligodendrocytes were seen in all the tracts of the white matter, except in the ventral part of the dorsal corticospinal tract (Fig. 6C), whereas no labeling was seen in the gray matter. These immunostained cells defined a subset of oligodendrocytes that are estimated, using anti-CV2 sera, to account for one-third of spinal cord oligodendrocytes, with a rostrocaudal gradient (Honnorat et al., 1998). Ulip6/CRMP5-
positive oligodendrocytes were rarely found in the forebrain: the gray matter or myelinated fibers. Similarly, Ulip2/CRMP2 has been shown to be expressed by a subpopulation of oligodendrocytes in adult brain (Ricard et al., 2000). In spinal cord and hindbrain and midbrain white matter, all oligodendrocytes stained by anti-Ulip6/CRMP5 antibodies were double stained by anti-Ulip2/CRMP2 antibodies, demonstrating that these two Ulip/CRMP proteins were coexpressed by certain oligodendrocytes (Fig. 6A,B). Interestingly, some Ulip2/CRMP2-expressing oligodendrocytes in the midbrain (Fig. 6B) and spinal cord, i.e., the ventral part of the dorsal corticospinal tracts (Fig. 6D), did not express Ulip6/CRMP5 (Fig. 6A,C). Because Ulip2/CRMP2 protein is expressed by only 40% of spinal cord oligodendrocytes (Ricard et al., 2000), three different subsets of oligodendrocytes can be distinguished in the spinal cord: one expressing both Ulip6/CRMP5 and Ulip2/CRMP2, another expressing only Ulip2/CRMP2, and a third expressing neither. On the other hand, it is noteworthy that, during ontogenesis, Ulip2/CRMP2 was detectable in oligodendrocytes at P15, whereas the earliest Ulip6/CRMP5-expressing oligodendrocytes appeared at P18 (Fig. 3B,E).

Inhibition of oligodendrocyte process extension by Sema3A: involvement of Ulip6/CRMP5 and Ulip2/CRMP2

To investigate the role of Ulip6/CRMP5 and Ulip2/CRMP2 in oligodendrocytes, we used highly purified adult rat brain oligodendrocytes, previously shown to express Ulip2/CRMP2 protein (Ricard et al., 2000) and shown, in the present study, to express Ulip6/CRMP5 protein (see Fig. 8A,B).

Because the cultured oligodendrocytes had been shown to have Sema3A binding sites (Fig. 7A–D) and to express the neuropilin-1 receptor sites (Fig. 7E–H), we examined their response to soluble Sema3A by incubating them for 24, 48, or 72 hr with or without Sema3A-conditioned medium. When cultured in the control medium, the cells displayed the morphological characteristics of oligodendrocytes, having round or ovoid cell bodies with a radiating array of thin tapering and branching processes, and expressing the oligodendrocyte marker, Rip (Fig. 8C); under these conditions, the oligodendrocytes could survive up to 20 d in culture. After 48 hr incubation in a Sema3A-conditioned medium, the oligodendrocytes showed significant loss of processes (Fig. 8D) compared with controls. Removal of Sema3A-conditioned medium led to oligodendrocyte process regrowth (Fig. 8E). To quantify oligodendrocyte arborization, we used a grid of concentric circles (Fig. 9) to define a BI (see Material and Methods). Freshly isolated purified oligodendrocytes initially had a mean BI close to zero (data not shown), then started to spontaneously send out processes with the time course shown in Figure 10A (control), with a maximal mean BI of 21.5 at 72 hr of culture. In Sema3A-conditioned medium, the BI decreased by 72% at 24 hr, 81% at 48 hr, and 88% at 72 hr compared with controls (p < 0.0001) (Fig. 10A). The Sema3A dose–response curve, determined using a range of dilutions of Sema3A-conditioned medium (undiluted to 1:100) diluted in control medium (Fig. 10B), showed a sigmoid shape consistent with a specific biologic effect. The half-effect, corresponding to a BI reduction of 50% (p < 0.005), was obtained at a 1:20 dilution (25 ng/ml of Sema3A) (Bagnard et al., 1998). When Sema3A-conditioned medium was added to oligodendrocytes grown 24 hr in control medium and displaying processes (mean BI of 12.2), no process alteration was observed at 2, 4, or 6 hr, but retraction of process extension was seen after 24 hr.
and was still observed after 48 hr (mean BI of 3.8) (Fig. 10). The Sema3A effect was totally reversed after removal of the Sema3A-conditioned medium and 72 hr incubation in control medium, the mean BI increasing to 20.8 (Fig. 10). It is noteworthy that oligodendrocytes cultured in Sema3A-conditioned medium expressed Rip, a marker of late stages of oligodendrocyte differentiation (Friedman et al., 1989) (Fig. 8).

Because it has been shown that Sema3A can induce apoptosis on developing or mature neurons (Shirvan et al., 1999; Bagnard et al., 2001), oligodendrocyte apoptosis was studied using propidium iodide staining. By this method, we failed to detect any apoptotic cells. Because oligodendrocytes seemed less numerous when cultured in Sema3A-conditioned medium than in control medium, we quantified both adherent and nonadherent cells. In Sema3A-conditioned medium, 22% of the cells were recovered in the culture medium, versus 6% in control medium (p < 0.01). By trypan blue staining, we noted no significant difference in cell death among these detached cells in Sema3A-conditioned medium (61%) versus control medium (44%). This decrease of adherent cells could be attributable to poorer adhesion of the Sema3A-treated oligodendrocytes caused by the absence of process arborization.

The effect of Sema3A signal on oligodendrocyte process extension was further investigated by blocking neuropilin-1 using antibodies directed against the MAM part of the receptor (Chen et al., 1998), which have been used successfully to block the effect of Sema3A on neurons (Bagnard et al., 2001). After 48 hr incubation in Sema3A-conditioned medium in the presence of anti-neuropilin-1 antibodies (4 μg/ml), the oligodendrocytes displayed a BI reduction of 25% compared with a reduction of 81% in the absence of antibodies (p < 0.001) (Fig. 10C). Furthermore, when VEGF-165, which has been proposed to antagonize Sema3A binding to neuropilin-1 (Miao et al., 1999), was added to Sema3A-conditioned medium at a concentration of 50 ng/ml, the BI was reduced by only 40% compared with 81% in the absence of VEGF-165 (p < 0.001) (Fig. 10C). These results indicated that the effect of Sema3A on oligodendrocytes was mediated by neuropilin-1.

To assess the role of Ulip2/CRMP2 and Ulip6/CRMP5 in transducing the Sema3A-induced inhibition of oligodendrocyte process extension, we used anti-Ulip2/CRMP2 antibodies to block Ulip2/CRMP2, as described by Goshima et al. (1995), and anti-Ulip6/CRMP5 or anti-CV2 antibodies to block Ulip6/CRMP5. After 48 hr incubation in Sema3A medium containing anti-Ulip2/CRMP2 antibodies at different concentrations (4, 8, and 20 μg/ml), a dose-dependent increase in the mean BI (BI = 21.2 at 8 μg/ml) was seen compared with oligodendrocytes grown in Sema3A-conditioned medium in the absence of antibodies (BI = 5; p < 0.001) (Fig. 10D). A significant block of the Sema3A effect on oligodendrocyte process extension was also seen using anti-Ulip6/CRMP5 antibodies (2, 4, and 8 μg/ml) (Fig. 10D) and anti-CV2 antibodies (data not shown). In contrast, anti-Ulip3/CRMP1 antibodies, recognizing specifically the Ulip3/CRMP1 protein (Fig. 1D), which is not expressed by oligodendrocytes, failed to block the Sema3A effect on oligodendrocyte process extension (Fig. 10D). In addition, oligodendrocytes cultured for 48 hr with anti-Ulip2/CRMP2 or anti-Ulip6/CRMP5 or anti-Ulip3/CRMP1 antibodies in control medium without Sema3A showed no morphological change. These results indicated that
Ulip2/CRMP2 and Ulip6/CRMP5 mediate the Sema3A effect on oligodendrocyte process extension.

DISCUSSION

The four previously described Ulip/CRMPs are highly expressed in the developing brain and are still expressed in the adult in some neurons (Minturn et al., 1995; Hamajima et al., 1996; Wang and Strittmatter, 1996; Byk et al., 1998) and oligodendrocytes (Kamata et al., 1998; Nacher et al., 2000; Ricard et al., 2000). During the course of this manuscript preparation, a fifth member with 50% homology with other Ulip/CRMPs was described in rat (CRAM) (Inatome et al., 2000) and mouse (CRMP5) (Fukada et al., 2000) brain. CRAM was discovered when antibodies against Zap-70, a Syk tyrosine kinase essential for T lymphocyte function, were found to cross-react with a protein kinase interacting with CRAM, and CRMP5 was discovered while studying molecules involved in regional specificity of the retina. Identifying an antigenic target in PND, we have cloned the fifth human Ulip/CRMP, characterized its cellular and anatomical distribution compared with Ulip2/CRMP2, and demonstrated its potential role in mediating the inhibitory effect of Sema3A on process extension of adult brain oligodendrocytes.

Molecular and cellular characterization of Ulip6/CRMP5

PNDs are characterized by autoimmune neuronal degeneration developing in patients with systemic cancer (Posner, 1997). Several autoantibodies associated with PND are directed against various nervous system antigens, including a group of proteins Ulip6/CRMP5 and Ulip2/CRMP5 mediate the Sema3A effect on oligodendrocyte process extension.

**Figure 5.** Expression of Ulip6/CRMP5 mRNA and protein in adult rat brain. Sagittal sections (14 μm) of the frontal cortex (A, B), hippocampus (C, D), or spinal cord (E, F) were hybridized with the Ulip6/CRMP5 riboprobe (A, C, E) or immunolabeled with anti-Ulip6/CRMP5 antibodies (B, D, F). Both mRNA (A, C) and protein (B, D) were expressed in some neurons of the frontal cortex (A, B) and hippocampus (C, D), especially in the infragranular layer (arrow). Both mRNA (E) and protein (F) were also expressed in oligodendrocytes of the spinal cord (arrowhead). Scale bar: A, 60 μm; B, 30 μm; C, 310 μm; D, 50 μm; E, 40 μm; F, 25 μm.

**Figure 6.** Expression of Ulip6/CRMP5 and Ulip2/CRMP2 mRNAs and proteins in oligodendrocytes. Sections (14 μm) of adult rat cerebellar peduncles were immunolabeled with both rabbit anti-Ulip6/CRMP5 antibodies (A) and rat anti-Ulip2/CRMP2 antibodies (B). All oligodendrocytes labeled by anti-Ulip6/CRMP5 antibodies expressed Ulip2/CRMP2 protein (arrow). A few oligodendrocytes expressing Ulip2/CRMP2 protein were negative for Ulip6/CRMP5 protein (arrowhead). Frontal sections (14 μm) of adult rat spinal cord were hybridized with the Ulip6/CRMP5 (C) or Ulip2/CRMP2 (D) riboprobes. Oligodendrocytes of the internal part of corticospinal tract expressing Ulip2/CRMP2 mRNA were negative for Ulip6/CRMP5 mRNA (arrows). Scale bar: A, B, 30 μm; C, D, 200 μm.
Ulip6/CRMP5 protein has also been described in adult brain (Fukada et al., 2000; Inatome et al., 2000). Expression of Ulip6 (also called CRMP5) in the adult brain, as seen with the other Ulip/CRMPs, corroborates previous reports of specific interactions between Ulip/CRMP isoforms (Wang and Strittmatter, 1997; Fukada et al., 2000; Inatome et al., 2000). Expression of Ulip1/CRMP4 (Byk et al., 1996). Expression of this protein in adult testis might also be related to the detection of Ulip1/CRMP4 in postmeiotic germ cells (Takeo et al., 1997; Kato et al., 1998).

In the brain, Ulip6/CRMP5 was highly expressed during development by almost all postmitotic neural precursors and by fasciculi of fibers of the white matter, suggesting a role in neuronal migration and axonal growth. The continued expression of Ulip6/CRMP5 in the adult brain, as seen with the other Ulip/CRMPs, in areas that retain postnatal neurogenesis (dentine granular layer, olfactory bulb, and rostral migratory stream), confirms the role of this member in neuronal migration/differentiation. Ulip6/CRMP5 mRNA was also detected in neurons of the hypothalamus, thalamus, cortex, amygdala, brainstem, and cerebellum. Because the protein was detected only in a few neurons in the cortex and amygdala, the neuronal expression of Ulip6/CRMP5 protein might be transient and required for synaptic plasticity.

In the adult brain, the most intense Ulip6/CRMP5 mRNA and protein expression was seen in oligodendrocytes in the pons, cerebellum, and spinal cord, a distribution similar to that seen for Ulip2/CRMP2 (Ricard et al., 2000), suggesting the coexpression of these proteins. Our demonstration, by double labeling, of the coexpression of these two Ulip/CRMPs in a subpopulation of oligodendrocytes corroborates previous reports of specific interactions between Ulip/CRMP isoforms (Wang and Strittmatter, 1997; Fukada et al., 2000; Inatome et al., 2000). Expression of Ulip1/CRMP4 protein has also been described in adult brain oligodendrocytes (Nacher et al., 2000). However, we have been unable to detect oligodendrocytic expression of this member by either in situ hybridization (Ricard et al., 2000) or immunohistochemistry with antibodies recognizing specifically recombinant Ulip1/CRMP4 (E. Charrier, unpublished data). Interestingly,
some Ulip2/CRMP2-positive oligodendrocytes, namely in the corticospinal tracts, did not express detectable levels of Ulip6/CRMP5, demonstrating the presence of subsets of oligodendrocytes differing in terms of Ulip/CRMP expressions. The presence of Ulip6/CRMP5 and Ulip2/CRMP2 in oligodendrocytes might be related to different functions of these cells, depending on their localization or degree of maturation. In fact, some Ulip/CRMP-expressing cells might be oligodendrocyte progenitors, retaining the capacity of migration/differentiation in adult brain (Zhang et al., 1999). However, differentiated oligodendrocytes expressed Ulip6/CRMP5, because loops around axons belonging to myelinating oligodendrocytes were labeled by anti-Ulip6/CRMP5 antibodies.

Interestingly, similar coexpression or lack of coexpression of Ulip2/CRMP2 and Ulip6/CRMP5 was seen during development. In the cerebellum, only Ulip2/CRMP2 was highly expressed in the external part of EGL containing the mitotic neural precursors, whereas both Ulip2/CRMP2 and Ulip6/CRMP5 were expressed in the internal part of the EGL, which contains the postmitotic migrating neuronal precursors. After migration, neuronal precursors in the EGL showed high expression of Ulip6/CRMP5 but low expression of Ulip2/CRMP2. In addition, during brain development, Ulip2/CRMP2 was expressed before Ulip6/CRMP5 in oligodendrocytes. Taken together, these results indicate that Ulip2/CRMP2 and Ulip6/CRMP5 either may have different roles in the intracellular signal cascade pathway in response to the same signal or may mediate different signals involved in the balance of positive and negative growth cues required in the regulation of neuronal migration/axonal growth and oligodendrocyte migration/process extension.

**Effect of Sema3A on oligodendrocytes: involvement of Ulip2/CRMP2 and Ulip6/CRMP5**

Ulip2/CRMP2 is reported to mediate Sema3A signaling axon guidance and collapse during development (Goshima et al., 1995; Pasterkamp et al., 1998a). Several lines of evidence suggest that Sema3A can act on oligodendrocytes in the adult brain: (1) neuropilin-1, a component of the Sema3A receptor complex (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), was shown to be expressed by oligodendrocytes, (2) addition of Sema3A to the culture medium dramatically reduced oligodendrocyte process extension, (3) removal of Sema3A resulted in restoration of process extension, and (4) the effect of Sema3A was blocked by anti-neuropilin-1 antibodies or VEGF-165, another neuropilin-1 ligand (Miao et al., 1999).
collapse is seen 10 min to 6 hr after Sema3A contact with neurons (Bagnard et al., 1998), in oligodendrocytes the Sema3A effect was seen after 24 hr. This time course is similar to that for the effect of phorbol ester on oligodendrocyte process extension (Stariha et al., 1997). Sema3A, via Ulip2/CRMP2 or Ulip6/CRMP5, may act on the dynamics of microtubules, leading to the decrease in process extension. Several results support this hypothesis: (1) microtubules are present in oligodendrocytes and reflect oligodendrocyte function (Lunn et al., 1997), (2) Ulip2/CRMP2 mediates the dynamics of microtubules (Gu and Ihara, 2000), and (3) protein kinase, which could phosphorylate the numerous phosphorylation sites of the Ulip/CRMPs, regulates oligodendrocyte process extension (Stariha et al., 1997) and can affect microtubule dynamics (Gotoh et al., 1991). Although these results show that oligodendrocytes are sensitive to Sema3A, other signals might also be mediated by Ulip/CRMPs (Wang and Strittmatter, 1997).

Functional significance of Ulip6/CRMP5 and Ulip2/CRMP2 expression in the adult brain

Oligodendrocytic expression of Ulip6/CRMP5 and Ulip2/CRMP2 could be crucial in mediating the signals involved in myelination, demyelination, and remyelination (at least in thepons, cerebellum, and spinal cord) in the normal and pathological brain. In demyelinating disorders, such as multiple sclerosis, before oligodendrocytes can remyelinate, they must extend and contact the demyelinated axons. The role of Ulip2/CRMP2 and Ulip6/CRMP5 in the response to signals, such as Sema3A, could be crucial in the reinitiation and regulation of process extension by surviving oligodendrocytes. High levels of Sema3A are expressed namely by fibroblasts in adult CNS scar tissue (Pasterkamp et al., 1998a, 1999), and Sema3A and Ulip2/CRMP2 expression has been shown to be upregulated in injured axons (Minturn et al., 1995; Pasterkamp et al., 1998b). Scar-derived Sema3A could thus affect both oligodendrocytes process extension and migration and axonal regrowth around the lesion in the injured brain. A physiologic role of Sema3A on oligodendrocytes should be also consider and could be assessed in Sema3A-deficient mice that are viable after birth (Taniguchi et al., 1997). In these mice, it would be interesting to study neuronal regeneration and myelination after brain injury.

The fact that neurons and oligodendrocytes may respond to similar signals, mediated by Ulip/CRMPs, opens new fields of investigation into the role of the neuron/oligodendrocyte interaction in axonal growth, in addition to the myelin-derived axonal growth inhibitors, MAG and N185/250 (Shibata et al., 1998; Buffo et al., 2000; GrandPrê et al., 2000). On the other hand, the specific increase of Ulip2/CRMP2 in Alzheimer’s disease (Gu et al., 2000) and the involvement of Ulip6/CRMP5 in PND patients emphasize the role of these proteins in neurological diseases. The tightly regulated spatial and temporal expression of the five Ulip/CRMPs is probably crucial in modulating the signal cascade pathways of positive/negative cues in both the developing and adult brain.

REFERENCES


