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SCO-spondin: a new member of the thrombospondin family secreted by the subcommissural organ is a candidate in the modulation of neuronal aggregation

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SUMMARY

A number of cues are known to influence neuronal development including growth factors, cell-adhesion molecules, components of the extracellular matrix and guidance molecules. In this study, we present molecular and functional evidence that SCO-spondin, a novel relative of the thrombospondin family, could also be involved in neuronal development by modulating cell aggregative mechanisms. SCO-spondin corresponds to glycoproteins secreted by the subcommissural organ (SCO), an ependymal differentiation of the vertebrate brain located at the entrance to the Sylvian aqueduct. A cDNA clone of 2.6 kb, isolated from a bovine SCO cDNA library, was shown to be specifically and highly expressed in the bovine SCO by in situ hybridization and was subsequently sequenced. Analysis of the deduced amino acid sequence reveals the presence of four conserved domains known as thrombospondin (TSP) type I repeats. To account for the homology with thrombospondins and F-spondin, this secreted glycoprotein was called SCO-spondin. Two potent binding sites to glycosaminoglycan (BBXB) and to cytokine (TXWSXWS) are also found in the TSP type I repeats. The deduced amino acid sequence exhibits three other conserved domains called low density lipoprotein (LDL) receptor type A repeats. The possibility of SCO-spondin involvement in neuronal development as a component of the extracellular matrix is discussed regarding these molecular features. The idea of a modulation of cell-cell and/or cell-matrix interaction is further supported by the anti-aggregative effect observed on cultured neuronal cells of material solubilized from Reissner's fiber. That Reissner's fiber, the condensed secretory product of the SCO present along the whole spinal cord can be a potent morphogenetical structure is an important concept for the analysis of the molecular mechanisms leading to spinal cord differentiation.

Key words: Development, Neuronal aggregation, SCO-spondin, Thrombospondin family

INTRODUCTION

The ependyma is a single-layered epithelium that covers the surface of the ventricles in the brain and the central canal in the spinal cord. During development, the ependyma is not simply the germinative layer generating the various neuronal cells but through their secretory function ependymal cells play a crucial role in morphogenetical events (reviewed by Sarnat, 1992; Del Bigio, 1995). The best documented secretory ependymal differentiation is that of the floor plate, a source of polarizing signals that control cell identity in the neural tube (Korzh, 1994). Numerous morphogenetical molecules have been reported to be secreted by the floor plate cells including: keratan sulfate proteoglycan; neural-cell adhesion molecule (N-CAM); cytokines (see Sarnat, 1992, for details); retinoic acid, a well-known morphogen (Wagner et al., 1990); BEN, a developmentally regulated antigen (Pourquié et al., 1990); F-spondin (Klar et al., 1992), favoring adhesion and neuritic outgrowth; and netrins (Kennedy et al., 1994; Serafini et al., 1994), having a chemoattractive effect on the commissural axons.

The most obvious ependymal secretion in the central nervous system (CNS) is a glycoproteinaceous material synthesized by the subcommissural organ (SCO) a structure located in the roof of the third ventricle at the entrance to the Sylvian aqueduct (reviewed by Oksche et al., 1993; Meiniel et al., 1996). The secretory activity of the SCO is detected early in the course of ontogeny (two and a half days in the chick embryo; Didier et al., 1992) but the SCO ependymal cells maintain their ability to secrete Reissner's fiber in adults.

Reissner's fiber (RF), which represents the condensing of the SCO secretion, is an intriguing thread running through the central canal of the spinal cord. This conserved structure from the vertebrate CNS has been shown to be present even in pro-

cordates from the moment a neural tube, exhibiting a central lumen, occurs in the course of phylogeny (Olsson, 1993). Using immunochemical techniques, it has been clearly demonstrated that the SCO in vertebrates is the source of synthesis for Reissner's fiber material as specific antibodies immunoreacted with both RF and SCO (reviewed by Oksche et al., 1993; Meiniel et al., 1996).

A morphogenetical activity of the SCO/RF complex has long been suspected (see Meiniel et al., 1996, for details) but in spite of a wide range of experimental work the exact functional significance of this secretory process in the CNS remains unclear.

The characterization of the cDNA encoding these secretions and the search for sequence homologies is essential to understand their putative function in the developing CNS. In this study, we describe a cDNA clone encoding an amino acid sequence that contains conserved domains (thrombospondin = TSP type I repeats and low density lipoprotein receptor = LDLr type A repeats) present in molecules of the extracellular matrix (ECM) playing a role in morphogenetical events. In addition, the putative biological activity of material solubilized from bovine Reissner's fiber was assessed in an in vitro model of cortical neuronal cells. This in vitro assay showed that this material can interfere with cell aggregation.

MATERIALS AND METHODS

cDNA library screening

A cDNA library was constructed in the vector λgt11 after extraction of mRNA from bovine embryonic subcommissural organ. A first positive clone, λRF101, isolated by immunoscreening using a polyclonal antibody raised against the SCO/RF secretion, had been previously characterized (Meiniel et al., 1995). This specific insert of 0.4 kb was used as a probe to rescreen this bovine SCO-cDNA library.

The library was plated at a density of 50,000 pfu/plate using the strain *Escherichia coli* Y1090. After overnight growth at 42°C, the plates were blotted on a nylon transfer membrane (Hybond N^+, Amersham). The filters were rinsed with 3× SSC, 0.1% SDS before hybridization. λRF101 probes were generated using the technique of random priming (Nona Primer Kit II, Appligene). The hybridization was carried out for 16 hours at 42°C in a 50% formamide solution (50% formamide, 0.75 M NaCl, 5% dextran sulfate, 50 μg/ml heparin, 1% SDS, 10 μg/ml poly(A), 50 μg/ml salmon sperm DNA). The filters were washed with 2× SSC, 0.1% SDS for 30 minutes at 42°C and with 0.2× SSC, 0.1% SDS for 30 minutes at 42°C, and then exposed at –80°C. The positive clones were purified and subcloned into pBluescript II SK DNA vector (Stratagene). The clone comprising the longest insert of 2.6 kb, λRF103, was completely sequenced.

DNA sequencing and analysis

Both strands of λRF103 were sequenced once and confirmed by sequencing one strand again and sequencing other overlapping clones on an Applied Biosystem 373A using the Dye Terminator technique.

Sequence comparison with GenBank, EMBL, Swissprot, NBRF was performed using the BISANCE service with the Fasta and Blast programs (Dessen et al., 1990). The Prosite database was used to search for protein sequence motifs (Bairoch and Bucher, 1994).

In situ hybridization histochemistry

In vitro transcription was performed using linearized DNA template λRF 103 inserted in pBluescript II SK plasmid. [^{35}S]dCTP labelled sense and antisense RNA probes were synthesized using T3 or T7 RNA polymerase promotors (according to the suppliers' instructions; Stratagene). Probe length was reduced to about 350 nucleotides by alkaline hydrolysis. Hybridization was performed on cryostat sections of bovine diencephalic roof containing the SCO for 12 hours at 50°C in a moist chamber (as described by Meiniel et al., 1995). The hybridization solution was at a final concentration of 50,000 cpm/μl. After washing, the sections were treated with RNAse A (20 μg/ml; Boehringer Mannheim) for 30 minutes at 37°C and washed twice in 2× SSC, 50% formamide for 1 hour at 55°C. The slides were subsequently dipped in LM1 nuclear emulsion (Amersham, UK). After 8 days, the radioautographs were developed and the sections were counterstained using toluidine blue.

Immunohistochemistry

Adjacent cryostat sections were treated with a polyclonal antibody raised against bovine Reissner's fiber whose specificity to the SCO/RF secretory glycoproteins has been previously determined (Meiniel et al., 1996). The second antibody labelled with peroxidase was revealed using 3-3′ diamino-benzidine tetrachloride (DAB) and hydrogen peroxide (H_2O_2).

Neuronal cultures

Cerebral hemispheres of 8-day-old chick embryos were dissected, mechanically dissociated and transferred to Ca^{2+}/Mg^{2+}-free PBS containing 0.25% trypsin for 15 minutes at 37°C. Fragments were pelleted by centrifugation at 200 ***g*** for 5 minutes in DMEM containing 10% fetal calf serum to remove trypsin. Individual cells were obtained by dissociation through a nylon sieve (pore size 48 μm) and were transferred into N2 medium (Bottenstein and Sato, 1979) (1:1 mixture of DMEM/F12 containing 20 nM progesterone, 30 nM sodium selenite, 100 μM putrescine, 2 mM glutamine, 33 mM D-glucose, 1 pM β-estradiol, 5 μg/ml insulin, and 50 μg/ml transferrin). For the aggregation assay 1.8×10^5 cells/cm^2, and for the adhesion assay 0.7×10^5 cells/cm^2 were, respectively, plated on poly-L-lysine (10 μg/ml)-coated 24-well plates and incubated in 10% CO_2/90% air at 37°C.

Reissner's fibre treatment

Isolation of Reissner's fibre (RF) from bovine spinal cord was performed as described previously (Monnerie et al., 1995). To test the effect of soluble RF-material on neuronal aggregation, 3 portions of 4 cm length RF per ml of N2 medium were incubated for 5 days at 37°C. After counting, and prior to seeding, cells were pelleted by centrifugation and the supernatant was replaced by N2 medium stock solution containing soluble RF-material (4-5 μg/ml) and adjusted to 1.8×10^5 cells/cm^2 for the aggregation assay or 0.7×10^5 cells/cm^2 for the adhesion assay.

Anti-RF treatment

Anti-RF antibodies (60 μg/ml), raised against bovine Reissner's fibre (Monnerie et al., 1995), and purified on Protein A using the 'Pierce ImmunoPure IgG Purification Kit' (Pierce, Paris, France), were added to the medium of control and treated cultures at the time of cell plating.

Cell count

Aggregation assay

On different days after plating, the number of neuronal aggregates was counted in randomly selected fields using phase-contrast microscopy. The aggregates were quantified using a reticule grid (1× 1 cm, subdivided into 100 squares) in the eyepiece of the microscope, with a magnification of ×125. Aggregates were counted if their size reached nearly 0.25 mm^2 according to the size of the reticule, and if they displayed at least one well-defined process, longer than one aggregate in diameter.

Adhesion assay

After incubation for 1 or 3 hours, cells were gently washed with PBS and the number of remaining cells was counted from three fields (at ×250) under phase-contrast microscopy. All conditions were set up in triplicate. The number of independent experiments is indicated by *n*.

Statistical analysis

All data were expressed as the mean ± s.e.m. of *n* independent observations and tested by analysis of variance (ANOVA; for independent series and with different parameters for each group), followed, where necessary, by the Newman-Keuls post-hoc test. The significance level was $P<0.05$.

RESULTS

Screening and characterization of λRF 103 cDNA clone

To isolate specific cDNA clones related to the secretory product of the SCO, we screened a SCO cDNA library prepared in the vector λgt11, using a cDNA (λRF 101) isolated previously by immunoscreening and whose specificity to the SCO secreted glycoproteins was demonstrated by means of in situ hybridization (Meiniel et al., 1995).

Screening of the SCO cDNA library using this 0.4 kb cDNA insert as a probe allowed us to identify several additional cDNAs overlapping λRF 101 among which clone λRF 103 contained a 2.6 kb insert. The respective position of the two clones is indicated in Fig. 3A. As the λRF101 cDNA insert hybridized with one major transcript of 13 kb in poly(A)$^+$ selected RNA from the embryonic bovine SCO, λRF 103 represents only a part of the encoding sequence.

The specificity of expression of λRF 103 transcripts in the SCO was confirmed by in situ hybridization histochemistry. Using the ^{35}S-antisense probe, a strong radioactive labelling is detected in the secretory ependymal cells that border the ventricular cavity as well as in cells of the same lineage located in the underlying hypendymal layer, intermingled with various neuro-glia and vascular elements (Fig. 1A and C). In the posterior commissure, a few reactive cells are arrayed along the septa (Fig. 1A and D). No labelling is observed either in the classical ependyma or in the nervous tissue. Using the ^{35}S-sense probe only evenly distributed silver grains were detected in these areas (Fig. 1B). Thus, λRF 103 mRNA are expressed at a high level in adult and embryonic bovine SCO but they are undetectable in the adjacent nervous tissue. In addition, the pattern of in situ hybridization superimposes that of immunore-

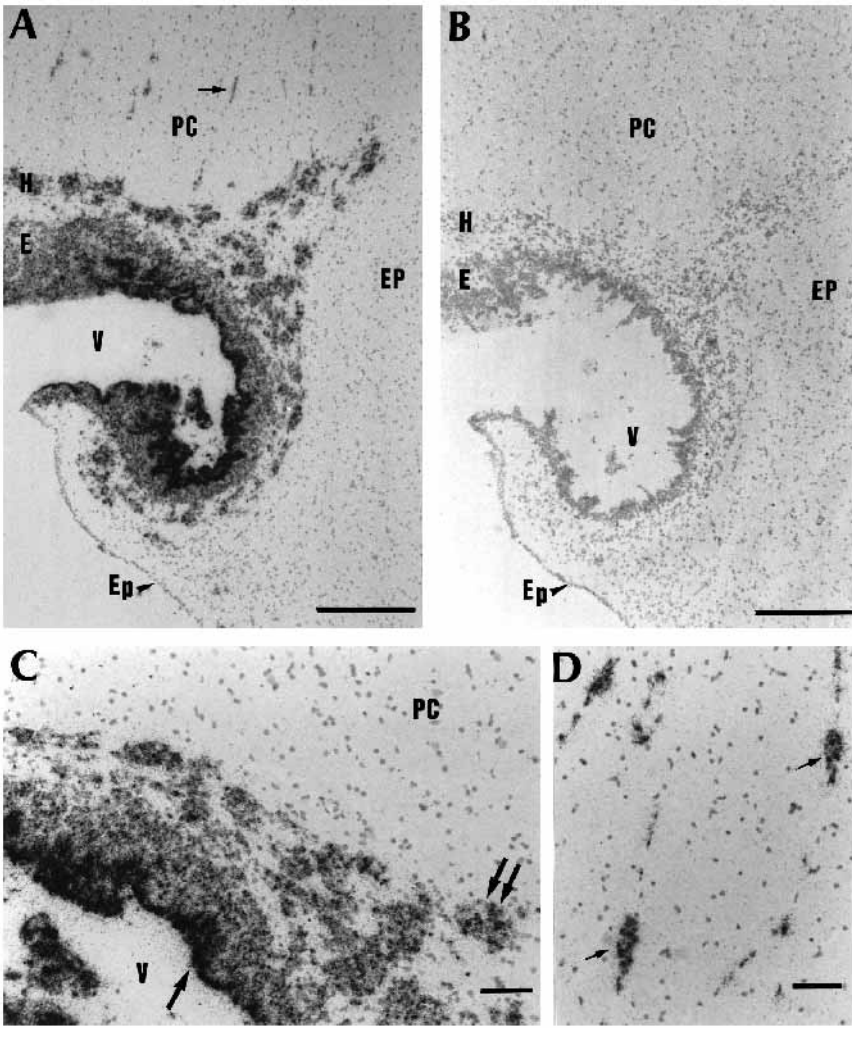


Fig. 1. Specific expression of λRF 103 mRNA in the adult bovine subcommissural organ. The same results are obtained in the 4- and 8-month-old embryonic bovine. (A) Sagittal section of the caudal end of the subcommissural organ hybridized with ^{35}S-λRF 103 antisense probe. Note the strong radioactive labelling in the secretory ependymal cells (E) lining the ventricular cavity (V). Hypendymal cells (H) in the underlying layer also contain silver grains. A few positive cells (arrow) are also visible in the posterior commissure (PC). In the nervous tissue of the posterior commissure (PC) and epithalamus (EP) as well as in the classical ependyma (Ep) silver grains are rare. Toluidine blue counterstaining. Bar, 500 μm. (B) Consecutive sagittal section hybridized with ^{35}S-λRF 103 sense probe. Silver grains are rare in the various components of the diencephalo-mesencephalic roof. Only the nuclei appear stained with toluidine blue. Bar, 500 μm. (C) High magnification showing strong radioactive labelling in the cytoplasm of the ependymal cells (arrow) lining the ventricule (V) and hypendymal cells arranged in 'rosettes' (double arrow). The nervous tissue in the posterior commissure is devoid of silver grains. ^{35}S-λRF 103 antisense probe; toluidine blue counterstaining. Bar, 100 μm. (D) High magnification of clustered positive cells (arrows) in the posterior commissure. ^{35}S-λRF 103 antisense probe; toluidine blue counterstaining. Bar, 100 μm.

activity obtained with antibodies directed against the SCO/RF secreted glycoproteins (Fig. 2).

λRF 103 cDNA corresponds to a deduced peptidic sequence exhibiting thrombospondin (TSP) type I repeats and low density lipoprotein (LDL) receptor type A repeats

The λRF 103 cDNA sequence exhibited no strong homology to any other known cDNA sequences in the GenBank database and it presented a single long open reading frame (Fig. 3A).

Translation of the open reading frame of the λRF 103 sequence into the amino acid sequence reveals that the predicted polypeptide contains two different types of repeated motifs, respectively, called thrombospondin (TSP) type I repeats and low density lipoprotein (LDL) receptor type A repeats.

The four TSP type I repeats present in the λRF 103 amino acid sequence correspond to domains of about 55 residues which can be aligned on the basis of conserved cysteine, tryptophan and arginine residues (Fig. 3B) and first identified in thrombospondin. Thus, the gene and the protein corresponding to λRF 103 is called SCO-spondin to reflect its high level of expression in the SCO and the presence of the TSP type I repeats indicating its relationship with thrombospondins and their relatives.

Furthermore, the λRF 103 predicted amino acid sequence contains a potent consensus site of attachment to glycosaminoglycan, a BBXB sequence (where B is a basic amino acid) found in a number of TSP type I repeats.

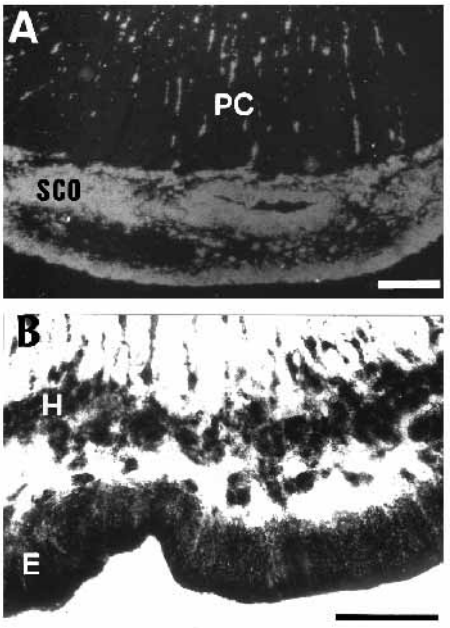


Fig. 2. Comparison of the in situ hybridization pattern with that of immunohistochemistry. (A) Strong radioactive labelling in the subcommissural organ (SCO) and posterior commissure (PC) after in situ hybridization with ^{35}S antisense probe. Dark field image. Bar, 500 μm. (B) Strong immunohistochemical labelling after exposure to an anti-RF antibody. The peroxidase activity is located in the secretory ependymal (E) and hypendymal (H) cells. Immunoperoxidase; no counterstaining. Bar, 500 μm. The same pattern is observed using monoclonal antibody $C_1B_8A_8$ (Meiniel et al., 1988).

The TXWSXWS sequence, a putative binding site to cytokine class II receptors may also contribute to the functional properties of SCO-spondin. In fact, the WSXWS sequence of the TSP type I repeat is found in the variant fibronectin type III repeat present in the receptors for several growth factors (ciliary neurotrophic factor = CNTF) and cytokines but the function of this motif remains unclear.

Three other repeated motifs correspond to cysteine-rich sequences of about 40 residues, first identified in the LDL receptor (Yamamoto et al., 1986) and called LDL receptor type A repeats (Fig. 3D). These consensus domains are often present in proteins of the extracellular matrix (ECM) such as heparan sulfate proteoglycan HSPG (cf. Kallunki and Tryggvason, 1992) or in morphoregulatory proteins resembling ECM such as nudel (Hong and Hashimoto, 1995) and in proteins of the complement cascade (Esser, 1994).

Finally, the presence of potent N-linked glycosylation sites corroborates previous lectin histochemical studies showing that the SCO/RF glycoproteins correspond to complex-type glycoproteins (see Oksche et al., 1993; Meiniel et al., 1996, for details).

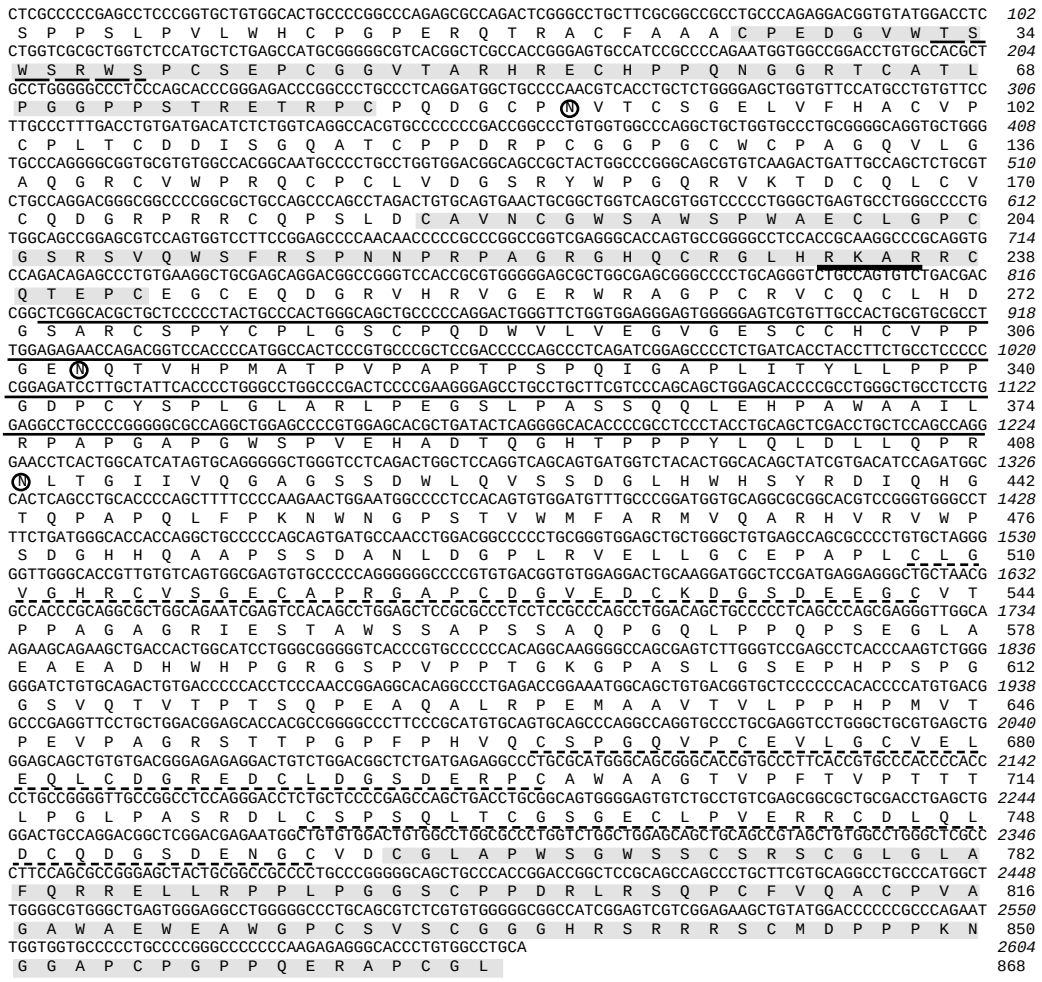
Comparison of the TSP type I repeats found in the proteins of the thrombospondin family

The consensus domain TSP type I repeats are present in a number of proteins (Fig. 3C), in addition to the glycoproteins encoded by the thrombospondin genes. The thrombospondin glycoproteins 1 and 2 each contain three TSP type I repeats (Lawler and Hynes, 1986) while C6-C9 proteins of the complement cascade exhibit only two of these conserved domains at their NH_2 and COOH termini (Haefliger et al., 1989). Properdin, the complement binding protein (Goundis and Reid, 1988) and F-spondin (Klar et al., 1992), mainly secreted by the floor plate cells, both contain six TSP type I repeats. In addition to these vertebrate proteins, one must also mention the circumsporozoite proteins (CS proteins), which mediate the binding of malarial sporozoites to host cells and TRAP (thrombospondin related anonymous protein) expressed in the blood stages of a human malaria parasite (*Plasmodium falciparum*) (Robson et al., 1988). In addition, two similar motifs are present in Unc-5, a modulator of axonal outgrowth in *Caenorhabditis elegans* (Hedgecock et al., 1990).

Compared to the various TSP type I repeats identified in the

Fig. 3. Analysis of λRF 103 cDNA sequence. (A) Nucleotide and deduced amino acid sequences. λRF101 clone that was used for cDNA library re-screening is underlined (continuous line). The thrombospondin (TSP) type I repeats are shaded and the low density lipoprotein (LDL) receptor type A repeats are underlined (dotted line). Potential sites of N-glycosylation (○), motif of cytokine receptor type 2 (– –), motif BBXB (—). (B) Alignment of the TSP type I repeats in SCO-spondin: residues identical in three or more repeats are shaded. (C) Comparison of the conserved TSP type I repeats found in SCO-spondin, thrombospondin 1, thrombospondin 2, properdin, F-spondin, CS protein, TRAP and Unc-5. The position of the first and the last residues of each repeat is indicated on the left. The quoted sequences were extracted from the Genbank and the EMBL databases. (D) Alignment of low density lipoprotein receptor type A repeats (LDLr) in SCO-spondin. The residues identical to the consensus domain are shaded.

A



B

	Residues nos	
1	26-81	C PEDGV WTS WSR WSPCS EPCG - G VTA - RH RECH - PPQ - NGGRT C ATLPGGPPSTRETRP C
2	185-243	C AVNCG WSAWSP WAECLGP CG SR SVQWSF R SPNN P RPAGRGHQ C RGLHRKARR C QTEP - C
3	762-813	C GLAP - - - - WSGWSS CSRS CG LG LAFQRRELLR - PP LP - GGS - C PPDRLRSQPC FVQA - C
4	813-867	C PVAGA WAEWEA WGP CSVS CG GG GHRS RRR SCMD PP PKNGGAP C PGPPQERAPC GLA

C

SCO-spondin	26-81	C-----W--WS-WS-CS--CG-G----R-R- C--PP---GG--C---------------C
	813-868	C-----W--W--W--CSV-CG-G- -R-R- C--P-P--GG--C----------C----
Thrombospondin 1	489-546	C-----W--WS-W--CSVTCG-G- -R----C--P-P--GG--C----------C-- --C
Thrombospondin 2	430-491	C---------WS-WS-WS-CSVTCG-G-- -R-R- C--P-P--GG--C----------C-- --C
Properdin	254-313	C-----WS-W--W--CSVTC--G-- -R-R- C--P-P--GG -C----------C-----C
F-spondin	615-665	C----WS-WS-CSVTCG-G- R-R----------G- C----------C-- --C
Plasmodium CS	346-390	WS-CSVTCG-G-- -R---- ----P--------- -----C-- --C
TRAP	244-291	C--W--WS-CSVTCG-G-- -R-R----- G C--- -----C-- --C
Unc-5	271-325	C---- WS-WS-WS--CS--C- R-R -C--P-P--GG--C----------C-----C

D

	Residues nos	
SCO-spondin	508-542	CL-GVGHRCVS--GECAPRGAPCDGVEDCKDGSDEE--GC
	665-699	CSPGQV-PCEVL-G-CVELEQLCDGREDCLDGSDER--PC
	725-759	CSPSQL-TCGS--GECLPVERRCDLQLDCQDGSDEN--GC
LDLr consensus		C-----F-C----G-CI-----CD---DC-DGSDE----C

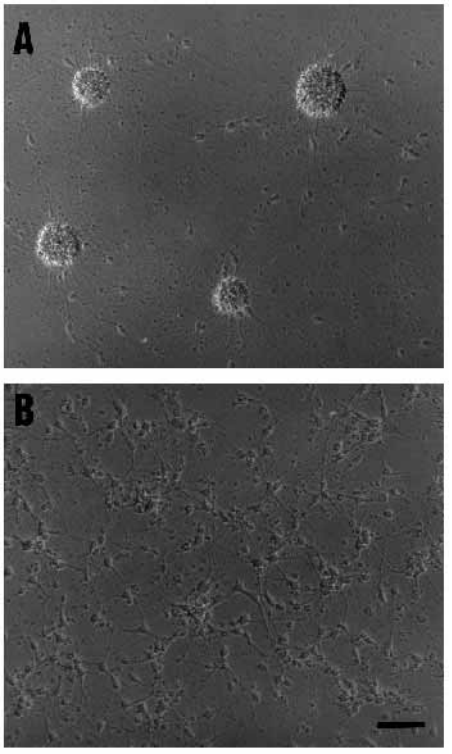


Fig. 4. Soluble RF-material displays anti-aggregative activity on neuronal cells plated at high density (1.8×10^5 cells/ml) on poly-L-lysine substratum. Relief contrast photomicrographs of primary neuronal cultures derived from cerebral hemispheres of 8-day-old chick embryos. Cells were grown for 7 days in the absence (A) or presence (B) of soluble RF-material. In control cultures, there are many neuronal aggregates whereas in treated cultures, neurons are mainly individual. Similar results were obtained on poly-L-lysine/collagen-coated plastic wells (data not shown). Bar, 100 μm.

molecules of the thrombospondin family, those in SCO-spondin share stronger homology with the motifs present in thrombospondins.

Soluble RF-material interferes with the aggregation of cortical neuronal cells in culture

To analyse whether the glycoproteins secreted by the subcommissural organ were in any way a factor involved in cell spreading, as is the case with thrombospondins, we tested the effect of solubilized Reissner's fiber (RF) proteins on an in vitro model of cortical neuronal cells. RF is held as the pure secretory product of the SCO and can be easily isolated from bovine spinal cords.

As shown in Fig. 4, by day 7 neuronal cells in control cultures aggregated strongly whereas in cultures treated with soluble RF-material, aggregates were rare and neuronal cells remained evenly distributed. In fact, the number of neuronal aggregates increased slightly with time in treated cultures but remained one third of that of controls (Fig. 5). This activity on cell spreading appears to be specific as in the presence of polyclonal antibodies raised against bovine RF, the anti-aggregative effect of soluble RF-material disappears (Fig. 6).

As shown in Fig. 7, there was no difference in the number of adhering neuronal cells either in control or soluble RF-material-treated cultures, 1 or 3 hours after plating.

DISCUSSION

The subcommissural organ is well known as a specialized area in the brain originating from an early differentiation of the ependymal lining. The secretory process of the SCO that gives rise to Reissner's fiber extending into the central canal of the spinal cord has been the aim of a number of studies but its exact functional role is still an enigma. By analogy with the floor plate that also represents a secretory differentiation of ependymal cells in the spinal cord, we have postulated a possible morphogenetical activity of the SCO/RF complex (reviewed by Meiniel et al., 1996).

In order to contribute to a better understanding of the function of the SCO/RF complex in the developing CNS, we have isolated and analyzed a 2.6 kb cDNA insert corresponding to a part of the cDNA that encodes the secretory SCO protein. Analysis of the predicted peptidic sequence indicates that the SCO secreted protein contains consensus domains already indentified in proteins of the ECM. Because of these homologies with proteins that can mediate adhesion and neuritic outgrowth in the developing CNS, such as thrombospondin 2 (O'Shea et al., 1991) and F-spondin (Klar et al., 1992) we called this protein SCO-spondin. Solubilized proteins from bovine Reissner's fiber (RF), that correspond to the proteins secreted by the SCO, are able to interfere with the aggregation of neuronal cells in an in vitro system. Both molecular features of SCO-spondin and in vitro activity of RF suggest that the SCO/RF complex could participate in morphogenetical events.

Specificity of λRF 103 cDNA and site of SCO-spondin synthesis

Much evidence supports the fact that SCO-spondin is mainly expressed in the SCO of vertebrates both in adult and embryo form. By in situ hybridization, it was proved that λRF 103 is highly specific to mRNA present in the secretory ependymal cells of the SCO since no other area in the brain was reactive. Furthermore, as the clone λRF 101 that was used as a probe

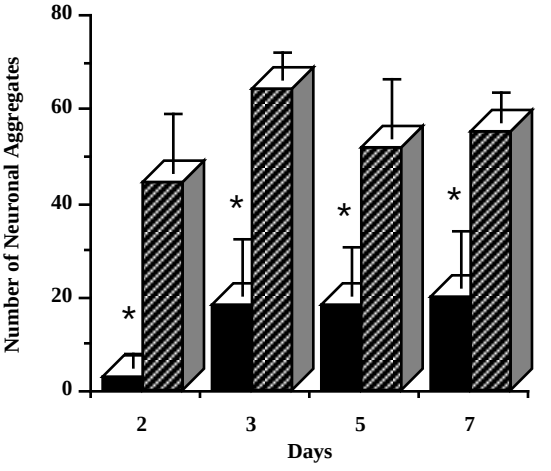


Fig. 5. Anti-aggregative effect of soluble RF-material on cortical neuronal cell aggregation during culture. Cells were plated in N2 medium only (striped bars) or N2 medium containing soluble RF-material (solid bars) and counted on days 2, 3, 5 and 7. Values are the mean ± s.e.m (n=3). *P<0.0001 versus controls (ANOVA).

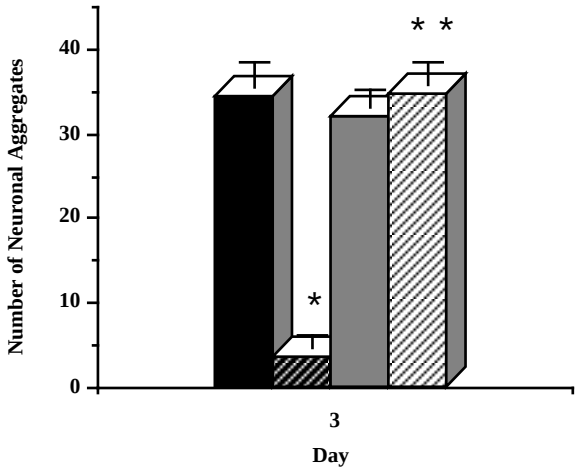


Fig. 6. Inhibition of the anti-aggregative effect of soluble RF-material using specific polyclonal antibodies. The number of neuronal aggregates was evaluated on day 3, in controls (solid bars), in the presence of soluble RF-material (broadly-striped bars), with anti-RF antibodies alone (shaded bars), or with soluble RF-material and anti-RF antibodies (thinly-striped bars). Affinity-purified polyclonal anti-RF antibodies were used at 60 μg/ml and added to the cultures concurrently with soluble RF-material. Values are the mean ± s.e.m (*n*=2). **P*<0.0001 versus controls; ***P*<0.0001 versus soluble RF-material (ANOVA).

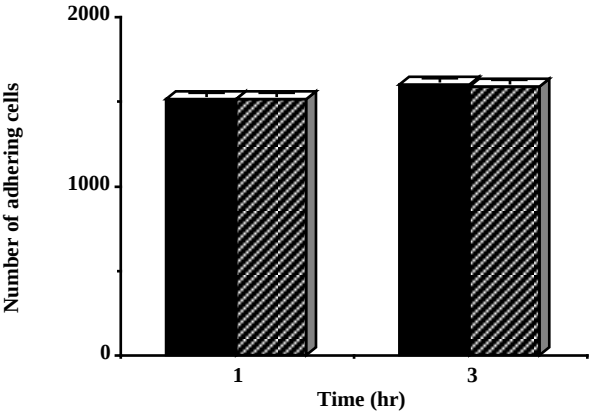


Fig. 7. Effect of soluble RF-material on neuronal cell adhesion. Cells were plated with (solid bars) or without (striped bars) soluble RF-material on poly-L-lysine-coated plates for 1 or 3 hours, washed with PBS and counted under phase-contrast microscopy. No differences were recorded between control and treated cultures. Values are the mean ± s.e.m (*n*=3).

for rescreening the SCO cDNA library was isolated by means of an antibody directed against Reissner's fiber, the transcripts revealed by the λRF 103 antisense probe encode secreted proteins that are a constituent part of Reissner's fiber. Conversely, the glycoproteins revealed after immunohistochemistry using anti-RF or $C_1B_8A_8$ monoclonal antibody probably correspond to SCO-spondin.

Although the SCO-spondin shares strong peptidic homologies with thrombospondins and F-spondin, the nucleic acid sequence of λRF 103 shows poor homologies with the cDNA encoding both thrombospondin and F-spondin proteins due to degeneration of the genetic code. The restricted distribution of SCO-spondin mRNA has to be underlined compared to that of glycoproteins belonging to the same family and expressed in the embryonic CNS such as thrombospondin II mRNA (Tucker, 1993) or F-spondin mRNA (Klar et al., 1992); the latter are more restricted to the floor plate but are also present in cells of the peripheral nerves, probably Schwann cells and in other tissues. Nevertheless, a systematic analysis of the sites of SCO-spondin expression at various developmental stages is needed to search for possible spatio-temporal variations in the pattern of SCO-spondin mRNA during early ontogenesis, as it has been shown that monoclonal antibodies specific to the SCO/RF secretion also reacted with the neuroectoderm in the chick embryo (Didier et al., 1992).

Both thrombospondins (reviewed by Frazier, 1991; Sage and Bornstein, 1991; Bornstein and Sage, 1994; Chiquet-Ehrismann, 1991) and F-spondin (Klar et al., 1992) are held as secreted glycoproteins with restricted distributions in the embryonic ECM. Immunohistochemistry has revealed that thrombospondin is particularly abundant in the developing central and peripheral nervous systems as well as in areas of connective tissue morphogenesis (O'Shea and Dixit, 1988).

Consensus domains and putative functions of SCO-spondin

The functional significance of the TSP type I repeats is still being debated but there is a lot of evidence to support the implication of this domain in cell adhesion. The VTCG sequence, present in TSP type I repeats of CS proteins, has been shown to confer the adhesive property of plasmodium to its target (Rich et al., 1990). In a similar way, this consensus sequence and its variants (VSCG, ATCG) appear to be important in the biological function of properdin (Prater et al., 1991), the complement binding protein. To date, only one VSCG sequence that could function as a potent binding site has been identified in the TSP type I repeats deduced from λRF 103 but this clone only corresponds to a part of the complete encoded protein.

Currently, thrombospondins have been most extensively studied in terms of their function. These molecules exhibit both adhesive and anti-adhesive properties in vitro and so it has been suggested that they play a unique role in modulating cell behaviour in vivo. In fact, several potent binding sites of thrombospondin to cellular receptors are suspected of interfering with cell-to-cell contact (Frazier, 1991) including an RGDA sequence which could bind to $\alpha_v\beta_3$ integrin, the VTCG sequences which could bind to CD36 or platelet gpIV, the carboxy-terminal domain which could bind to the heterodimeric 105/80 kDa receptor, calcium-dependent, and the BBXB sequences which could bind to proteoglycans. The exact function of SCO-spondin remains to be determined but the presence in the λRF 103 amino acid sequence of TSP type I repeats containing a BBXB sequence that can function as a glycosaminoglycan binding site gives support to the idea that SCO-spondin may act as an extracellular modulator of cell-cell surface and/or cell-matrix interaction just like thrombospondins. Thrombospondins form complexes with proteoglycans (Prater et al., 1991) and this type of interaction may serve to strengthen thrombospondin binding or alternatively could prevent binding of CD36 thus allowing for the modulation of adhesion at these sites (Frazier, 1991).

In our in vitro assay, soluble RF-material exhibits an anti-aggregative effect on cortical neuronal cells that could be

inhibited using polyclonal antibodies raised against RF. Nevertheless, production of peptides from SCO-spondin is now needed to demonstrate that SCO-spondin is indeed involved in this anti-adhesive activity and to determine the functional domains implied in this mechanism.

Extracellular proteins that modulate cell-cell and cell-matrix interactions include molecules that foster both attachment and spreading in most cells such as thrombospondins, SPARC, tenascins (Sage and Bornstein, 1991; Chiquet-Ehrismann, 1991). Thus, in other bioassays, it is not impossible that RF material can exhibit an aggregative effect. At the molecular level we do not completely understand the specific associations that take place between these anti-adhesive proteins and their targets and of these only thrombospondin exhibits TSP type I repeats. All these proteins are known to interfere with cell attachment, to activate the intracellular signaling pathway, to influence Ca^{2+} ion flux, to present or sequester growth factors and cytokines and to modulate extracellular protease activity (Sage and Bornstein, 1991). The presence of a potent binding site to cytokines, TXWSXWS, in the λRF 103 amino acid sequence indicates that SCO-spondin could also participate in the regulation of growth factors delivery. Such a mechanism could explain the neuronal survival response observed in the presence of RF in a mixed neurone/glia assay (Monnerie et al., 1995).

The exact functional significance of the LDL receptor type A repeats present in molecules of the ECM is not yet completely understood. Recently, Hong and Hashimoto (1995) have suggested that in the nudel protein these motifs could bind extracellular proteases or their zymogens. Regulation of extracellular protease activity is known to be important in the developmental process (cf. Hecht and Anderson, 1992).

Thus, the SCO-spondin can be regarded as a large modular protein of the ECM exhibiting multiple potent sites of protein-protein interaction. Since SCO-spondin could bind to several extracellular proteins, which themselves could interact with their own cellular receptors, this molecule probably has multiple indirect means of cellular interaction.

SCO-spondin and CNS development

By immunochemical techniques, extracellular secreted SCO-spondin has been located in the leptomeningeal spaces close to the hypendymal vessels present in the posterior commissure (basal secretion) as well as in Reissner's fiber (ventricular secretion). In addition, the secreted glycoprotein is suspected of being soluble in the cerebrospinal fluid (CSF; reviewed by Oksche et al., 1993; Meiniel et al., 1996).

Whether SCO-spondin has a 'local' effect and may: (i) act on the differentiation of the commissural axons of the posterior commissure when secreted basally; or (ii) maintain the opening of the central canal of the spinal cord when secreted apically forming Reissner's fiber has yet to be determined. A more 'general' effect of SCO-spondin conveyed by the CSF on the differentiation of neuronal cells, in connection with the biological activity observed on our in vitro systems, may also be plausible. Another possibility could be that the TSP type I repeats are involved in aggregation of various SCO-spondin molecules to form Reissner's fiber.

Keynes and Cook (1990) have put forward a spectrum of molecules ranging from the highly adhesive glycoproteins N-CAM and N-cadherin to inhibitory glycoproteins that mediate cellular repulsion. Both adhesive and anti-adhesive mechanisms involving attractive and repulsive interactions between cells and their ECM may be important for cell migration and directional guidance of axon growth during neural development (Keynes and Cook, 1990; Edelman et al., 1990; Faissner and Kruse, 1990). Participation of SCO-spondin in these regulatory processes remains an interesting hypothesis.

Nevertheless, SCO-spondin is also expressed and synthesized during adult life and like thrombospondins that interfere with several biological events (angiogenesis, platelet aggegation, tumor growth, wound healing, neural crest cell migration and aggregation, peripheral nerve development, growth and process formation (reviewed by Sage and Bornstein, 1991) SCO-spondin may have a wide range of biological activities that have yet to be determined.

This study contributes to the identification of a new member of this growing thrombospondin family of extracellular glycoproteins that can modulate cell-cell and/or cell-matrix interactions during ontogenesis of the CNS. Knowledge of the whole SCO-spondin sequence will provide additional information regarding other conserved domains that may contribute to the function of this protein. In addition, new in vitro assays must be developed to examine the molecular interactions that take place between SCO-spondin and neurons in differentiation. Both aspects must provide help in understanding the exact role of SCO-spondin in morphoregulatory processes.

The nucleotide and peptide sequences data reported in this paper will appear, respectively, in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X93922. Werner Lehmann was supported by a postdoctoral fellowship from the DAAD Grant D 94/ 16861.

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