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REGULAR ARTICLE

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Effect of synthetic peptides derived from SCO-spondin conserved domains on chick cortical and spinal-cord neurons in cell cultures

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Abstract SCO-spondin is a newly identified protein, strongly expressed in the subcommissural organ (SCO), an ependymal differentiation of the brain. When secreted into the cerebrospinal fluid at the entrance to the Sylvian aqueduct, it condenses and forms Reissner's fiber. Several conserved domains have previously been characterized in SCO-spondin, e.g., thrombospondin type 1 repeats (TSRs), low-density lipoprotein receptor (LDLr) type A repeats, and epidermal-growth-factor-like domains, which are potent sites of protein-protein interaction. To clarify the role of this protein on neuronal development, we have tested the effect of oligopeptides, the sequences of which include highly conserved amino acids of TSRs, LDLr type A repeats and a potent site of attachment to proteoglycan, on cortical and spinal-cord neurons in primary cell cultures. One of these peptides (WSGWSSCSRSCG), corresponding to a SCO-spondin TSR sequence, markedly increases adhesivity and neuritic outgrowth of cortical neurons and induces an opposite effect on cortical and spinal-cord neuronal aggregation. These effects are specific, as no response is observed with the scrambled sequence of this peptide. Another peptide (WGPCSVSCG) is only slightly active on adhesivity and neuritic outgrowth of cortical neurons and has no effect on spinal-cord neurons. Peptides derived from other conserved domains of SCO-spondin are not effective under our experimental conditions. Thus, SCO-spondin may be responsible for at least a part of the effects previously observed on neuronal cells cultured in the presence of Reissner's fiber. In addition, SCO-spondin seems to interfere with neuronal development and/or axonal guidance during ontogenesis of the central nervous system in modulating side-to-side interactions and neuritic outgrowth.

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Introduction

SCO-spondin, first identified in cattle (Gobron et al. 1996), is a secreted protein of the central nervous system (CNS) and is strongly expressed in the subcommissural organ (SCO), an ependymal differentiation located at the entrance to the Sylvian aqueduct (see Oksche et al. 1993; Meiniel et al. 1996). This protein, when released into the cerebrospinal fluid (CSF), contributes to the formation of Reissner's fiber (RF), a thread-like structure running along the central canal of the spinal cord. RF has a remarkable phylogenetic constancy; it has been described in all subphyla of chordates (Olsson 1993), and the SCO/RF secretory process appears early during ontogeny (Schoebitz et al. 1986; Didier et al. 1992, chicken; Meiniel et al. 1990, cattle).

We have shown that RF modulates cell adhesion mechanisms and increases in vitro survival of both primary cortical and spinal-cord neuronal cells (Monnerie et al. 1995, 1996, 1997a, b). SCO-spondin present in RF has been partly sequenced; this protein exhibits molecular features that could account for at least some of the effects observed when developing neurons are exposed to RF. SCO-spondin shares homologies with molecules expressed in the nervous system, including the extracellular matrix (ECM) proteins thrombospondin 1 and 2 (Frazier 1991; Bornstein 1995), F-spondin, a morphogenetic molecule expressed in the floor plate, another secretory ependymal differentiation present in the developing spinal cord (Klar et al. 1992), the transmembrane proteins semaphorin F and G (Adams et al. 1996), and the *Caenorhabditis elegans* protein UNC-5 (Leung-Hagesteijn et al. 1992). All these molecules exhibit several conserved domains known as thrombospondin type 1 repeats (TSRs) and are suspected of playing a crucial role in cell adhesivity and neuritic outgrowth (Neugebauer at al. 1991;

O'Shea et al. 1991; Leung-Hagesteijn et al. 1992; Osterhout et al. 1992; Adams et al. 1996).

The putative function of thrombospondins has been extensively studied in various biological systems (for a review, see Adams et al. 1995), and it is generally accepted that TSRs mediate binding interactions of thrombospondin with other molecules in a variety of cell types (Prater et al. 1991). Thrombospondin has been shown to promote process outgrowth of both peripheral and central nervous system neurons (Neugebauer at al. 1991; Osterhout et al. 1992; De Freitas et al. 1995), and a 140-kDa fragment containing TSRs is known to be able to promote both adhesion and neurite outgrowth in vitro (O'Shea et al. 1991). Likewise, the recombinant F-spondin of 116 kDa, which contains TSR motifs, also increases both cell adhesion and neuritic outgrowth (Klar et al. 1992). Interestingly, several conserved domains have been identified in bovine SCO-spondin, including TSRs, low density lipoprotein receptor (LDLr) type A repeats, and epidermal-growth-factor (EGF)-like repeats (Gobron et al. 1996).

In order (1) to analyze whether SCO-spondin could account for at least some of the various RF effects observed previously in vitro and (2) to identify sequences that could mediate adhesion and neurite outgrowth, we have tested the effects of several peptides containing the most conserved amino acids of the consensus TSR and LDLr sequences of SCO-spondin on chick cortical and spinal-cord neurons in cell culture. These sequences are putative sites of SCO-spondin interaction with other proteins and so might participate in these processes (Gobron et al. 1996).

Materials and methods

Cell culture systems

Cortical neurons

Neuronal cultures were obtained from 8-day-old chicken embryos, a favorable stage from which to maintain cortical neurons in culture. Cerebral hemispheres were stripped of meninges, cut into small pieces, and enzymatically dissociated with 0.25% trypsin (Boehringer, Mannheim, France) in Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline (PBS) for 15 min at 37°C. The cells were centrifuged at 200*g* for 5 min in Dulbecco's modified Eagle's medium (DMEM; Boehringer) containing 20% fetal calf serum (FCS; Boehringer) for trypsin inactivation. The cells were then filtered through a nylon sieve (pore size: 48 µm) and collected in a serum-free, chemically defined medium consisting of a 1:1 mixture of DMEM and Ham's F12 (Boehringer) supplemented with glutamine (4 mM), glucose (33 mM), penicillin G (50 U/ml), streptomycin sulfate (50 µg/ml), and the N2 supplements of Bottenstein and Sato (1979); putrescin (100 µM), sodium selenite (30 nM), human transferrin (50 µg/ml), progesterone (20 nM), insulin (5 µg/ml), and β-estradiol (1 pM). All N2 supplements were purchased from Sigma (Saint-Quentin-Fallavier, France). Cells were plated at a density of 1.5×10^5 cells/cm^2 onto 24-well plastic plates. Alternatively, for experiments with low-density cultures, cells were plated at a density of 7.5×10^4 cells/cm^2 onto 24-well plastic plates. For some experiments, plastic plates were coated with either fibronectin (24 µg/ml) (Boehringer) or thrombospondin (20 µg/ml; Boehringer). The cultures were incubated at 37°C and 10% CO_2 in air. The medium remained unchanged throughout the experiment. We had shown previously that these cultures consisted of more than 95% neuronal elements (Monnerie et al. 1996).

Spinal-cord neurons

Spinal cords from 6-day-old chicken embryos (stage determined as favorable for the maintenance of spinal-cord neurons in culture) were dissected out, cleaned of their meningeal membranes, and minced into small pieces in Ca^{2+}-free and Mg^{2+}-free PBS. After incubation with 0.25% trypsin (Boehringer) for 10 min at 37°C, the tissue was centrifuged at 200*g* for 4 min in growth medium containing 20% FCS (Boehringer) to stop trypsination. The cells were then dissociated by repeated trituration with a Pasteur pipette having a fire-polished tip and resuspended in serum-free, chemically defined medium, as described above.

Cells were plated at a density of 7.5×10^4 cells/cm^2 onto 24-well plastic plates. The cultures were incubated at 37°C and 10% CO_2 in air. The medium was not changed throughout the experiment. We had previously demonstrated that the cell population under investigation consists of at least 93% neuronal elements (Monnerie et al. 1997b).

Immunofluorescence analysis

To identify neuronal cells in the cultures, we used an antibody raised against the 68-kDa neurofilament protein. Cultures were fixed in ethanol/ acetic acid (95:5) for 4 min, washed with PBS, and incubated for 30 min in PBS containing 2% bovine serum albumin (BSA). Immunostaining of neuronal cells was performed for 2 h at room temperature (RT) with mouse monoclonal anti-68-kDa neurofilament protein (anti-NF, Sigma) diluted 1:10 in PBS/BSA. Cultures were then washed with PBS and incubated with tetramethylrhodamine-isothiocyanate-conjugated sheep anti-mouse IgG antibodies (Sigma) diluted 1:100 in PBS/BSA for 2 h at RT. After being washed, the immunostained cells were examined under a Leica microscope equipped with an epifluorescence system.

Astrocytes were labeled by using glial fibrillary acidic protein (GFAP) according to the same procedure as described for neurons. Rabbit antiserum to GFAP (Sigma) was added at 1:80 to the blocking buffer for 2 h at RT. Visualization was performed by using fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG antibodies (Sigma) diluted 1:100.

Experimental procedures

The amino-acid sequences of the oligopeptides, WSGWSSCSRSCG (pep1), WGPCSVSCG (pep2), RKAR (pep3), DCKDGSDE (pep4), and a scrambled sequence of the former, SSCRSGCWGSSW (pep1 S), were purchased from Synt:em (Nîmes, France). An aliquot of 6 µl of each peptide (25 µg) was spotted onto the center of each well of 24-well plastic plates and incubated at 37°C. After 4 h, the plates were washed twice with culture medium, and neurons were added to the dishes as described above and then examined (on the indicated days after plating) for morphological appearance and morphometric analysis.

Alternatively, for experiments with ECM proteins, 24-well plastic plates were previously coated with either fibronectin (24 µg/ml) or thrombospondin (20 µg/ml) prior to the application of 6 µl of the corresponding peptide.

Soluble heparin was added to the culture medium at either 40 µg/ml or 80 µg/ml at the time of cell plating in both control and pep1-treated cultures. For experiments with substrate-coated heparin, both heparin (40 µg/ml) and pep1 were added in the same spot and incubated for 4 h at 37°C. The plates were then washed twice with culture medium, and neuronal cells were plated directly onto the plates.

For experiments to test the effect of pep1 and pep2 in solution, peptides were added to the culture medium at the time of cell plating, at concentrations ranging from 50 µg/ml to 500 µg/ml.

Quantitative analyses

Aggregation assay

To quantify the effect of peptides on neuronal aggregation, we counted the number of neuronal cell aggregates by examining phase-bright cells in fields randomly selected with a reticule grid (1×1 cm, subdivided into 100 squares) in the eyepiece of the microscope, at a magnification of ×125. Aggregates were counted when their size reached nearly 0.25 mm^2 corresponding to the size of the reticule, and when they displayed at least one well-defined neurite bundle, longer than one aggregate diameter. Some 2.5% of the total surface of the well was examined. The number of independent experiments is indicated by *n*.

Adhesion assay

Plastic plates (24-well) were coated with 6 µl pep1, pep2, pep1 S, or BSA and were then incubated for 4 h at 37°C. After two washes with culture medium, 1.5×10^5 freshly dissociated cells were seeded into each well. After 1 h of incubation at 37°C, the cells were gently washed with PBS to remove unattached cells, and the number of adherent cells was counted from three fields (at ×250) under the phase-contrast microscope.

Alternatively, peptides were bound to individual wells by nitrocellulose dissolved in methanol as described by Lagenaur and Lemmon (1987). The procedure then followed the above-described protocol. Experiments were performed in triplicate and repeated three times.

Neurite extension from cortical neurons

Dissociated cortical cells were added to either peptide-coated 24-well plastic plates or untreated plastic plates. After 5 days (stage of optimal difference between control and treated cultures), neurites were scored as present if the processes were longer than one aggregate diameter. The number of neurites per aggregate and the length of the longest neurite per aggregate were determined from three regions of each well and from at least three replicate cultures, provided the aggregates were of the same size.

Neurite extension from spinal-cord neurons

To examine sprouting of neurons, dissociated cells were added to either peptide-coated 24-well plastic plates or untreated plastic plates. After 7 days (stage of optimal difference between control and treated cultures), neurites were scored as present if they were longer than one cell diameter. Experiments were performed at least three times.

Statistical analysis

Data were expressed as the mean±SEM of *n* independent experiments. For all experiments, we statistically compared control groups and peptide-treated groups by using 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

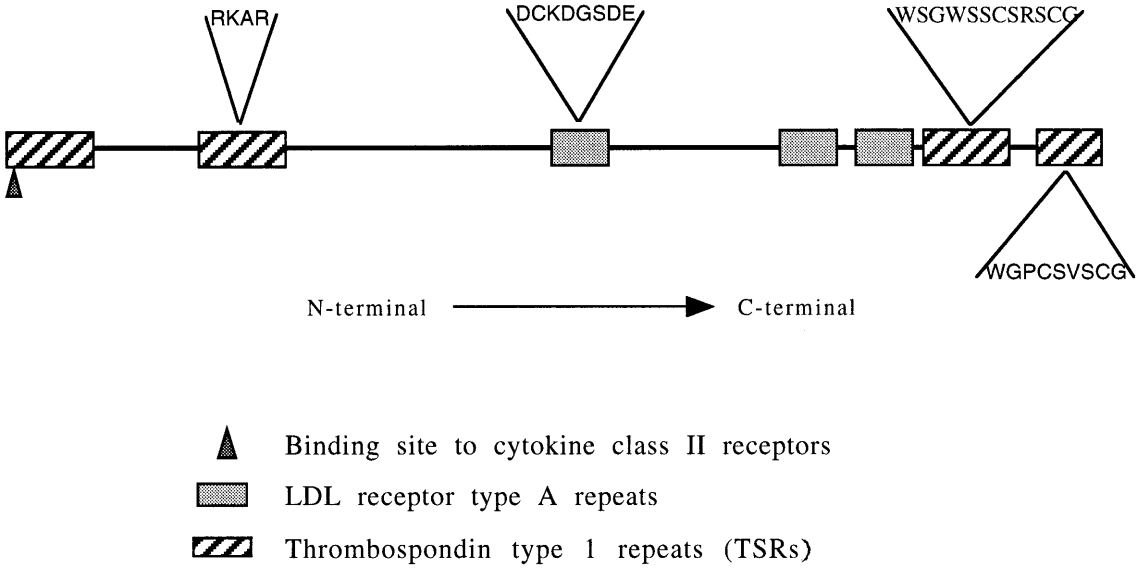
Several peptides deduced from the partially characterized SCO-spondin sequence were first tested on neuron-enriched cultures of dissociated embryonic chick cortical and spinal-cord cells. The peptides chosen included (1) two sequences within two different thrombospondin type 1 repeats (TSRs), (2) one sequence present in one of the LDLr type A domains, and (3) one glycosaminoglycan-binding sequence also identified in a number of heparin-binding proteins (Cardin and Weintraub 1989) (Fig. 1). These correspond to highly conserved amino acids of the TSR and LDLr consensus.

Effect of SCO-spondin-derived peptides on cortical neurons

TSR-derived peptides

Pep1: WSGWSSCSRSCG. On plastic alone, cortical neurons typically formed aggregates from which extended very few neurites (Fig. 2A). Control cells seeded in the

Fig. 1 Schematic drawing of the SCO-spondin sequence (AC number X93922) including the position and sequence of the four peptides tested. The thrombospondin type 1 repeats (*TSRs*) are *striped*, the low-density lipoprotein (*LDL*) receptor type A repeats are *shaded*, and the binding site to *cytokine class II receptors* is represented by a *triangle*. Amino acid sequences correspond to the peptide position within a specific domain



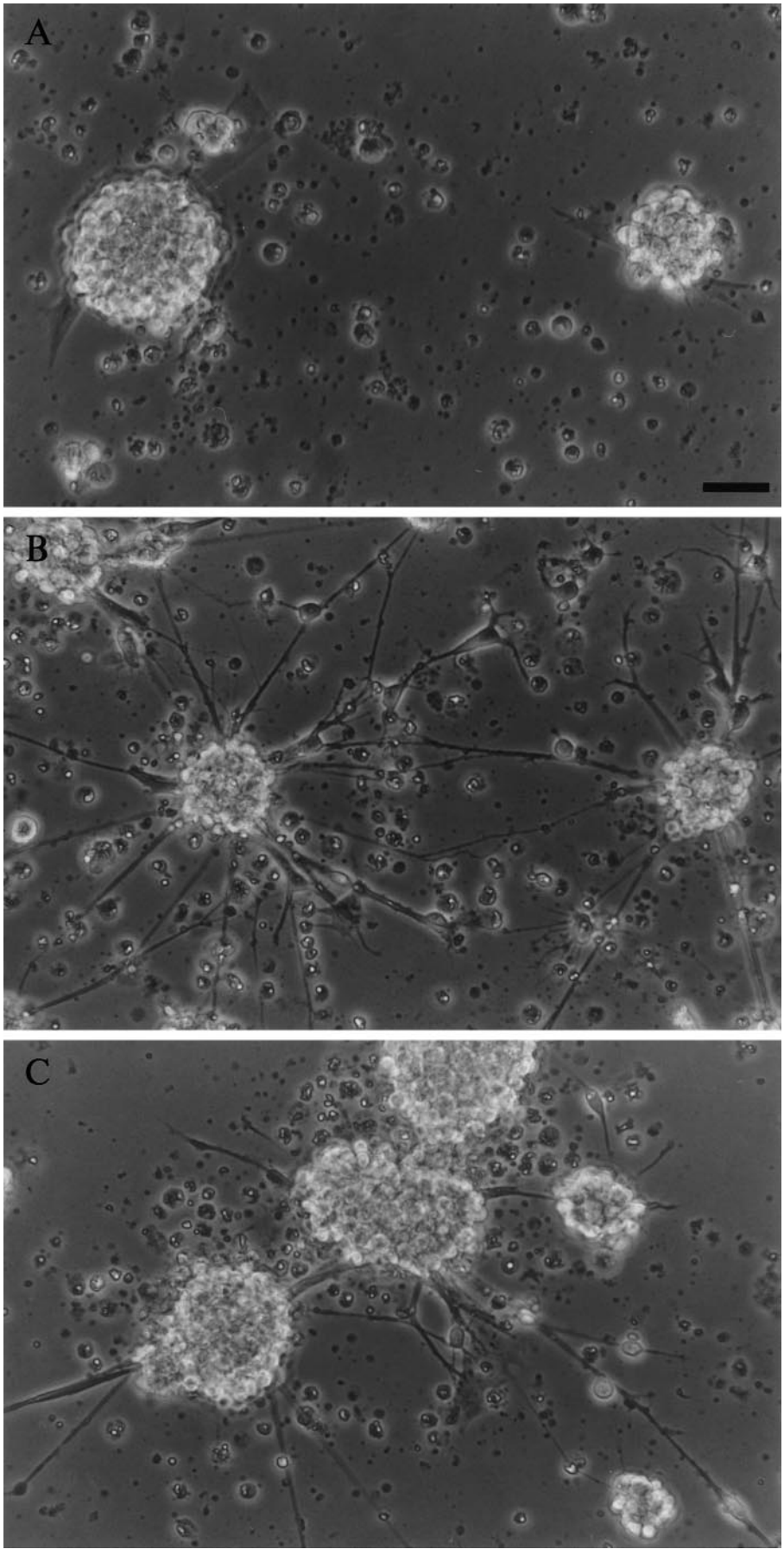


Fig. 2A–C Effect of pep1 and pep2 on the morphology of cortical neurons. Phase-contrast micrographs of cells grown for 5 days in the absence of any substrate. **A** Control culture in which neuronal cells constitute aggregates without neuritic outgrowth. **B** In the presence of pep1, numerous neuritic processes extend from the neuronal aggregates. **C** In comparison with pep1, neuritic outgrowth is less well-developed in the presence of pep2. *Bar* 50 µm

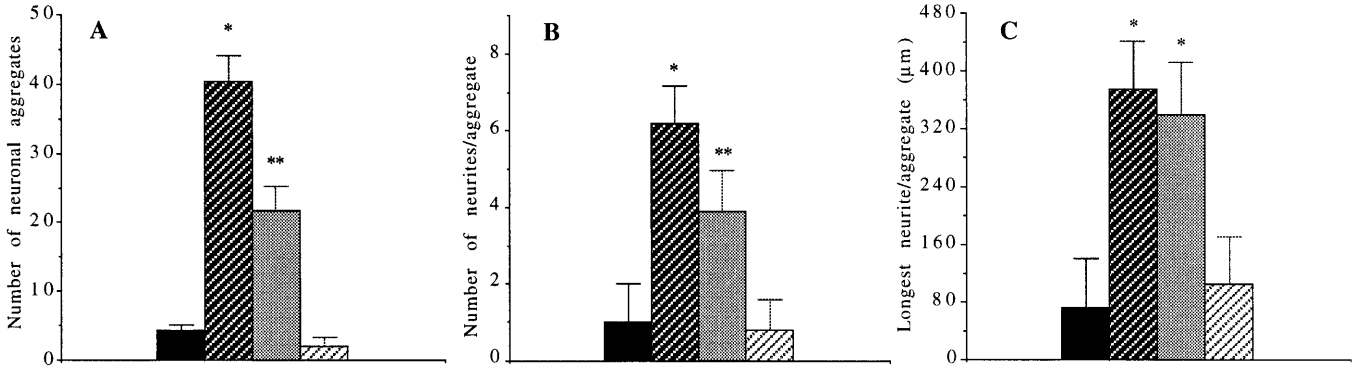


Fig. 3A–C Quantitative analysis of cortical neuronal aggregates in the presence of pep1 or pep2. Cells were grown for 5 days in N2 medium only (*solid bars*), in the presence of pep1 (*broadly striped bars*), in the presence of pep2 (*shaded bars*), or with BSA (*narrowly striped bars*). The number of neuronal aggregates (**A**), the average number of neurites per aggregate (**B**), and the average length of the longest neurite per aggregate (**C**) are shown. Values are the mean±SEM (n=3). *P<0.0001 vs controls; **P<0.001 vs controls (ANOVA)

absence of any peptides were gradually lost from the plastic substrate during the first 5 days of culture. In contrast, on the spot area where pep1 had been coated, aggregated neurons were mostly connected by long thick bundles of neurites within 5 days in culture (Fig. 2B). Furthermore, cells adhered well to the pep1-coated substrate, and no aggregate detachment was detected. The number of neuronal aggregates (Fig. 3A) increased 9.3-fold from control cultures to cultures growing on pep1 (4.33±0.47 to 40.33±4.64), and both the number of neurites per aggregate (Fig. 3B) and the length of the longest neurite of an aggregate (Fig. 3C) were significantly enhanced in the presence of pep 1. Neuronal cells adhered poorly to BSA-coated plastic wells.

Pep1 S: SSCRSGCWGSSW. To make sure that the primary amino-acid sequence was essential for the functional activity of pep1 and that biological activity was not merely the result of the peptide net charge or amino-acid composition, we tested the scrambled peptide (pep1 S) at the same concentration (25 µg/spot) as for the "native" unscrambled peptide (pep1). No significant increase in cell adhesion or neurite outgrowth was observed in the presence of pep1 S (data not shown).

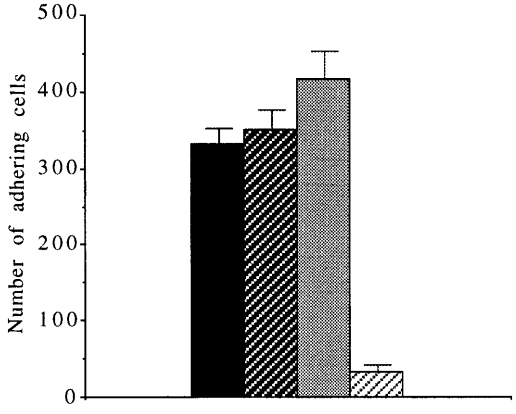
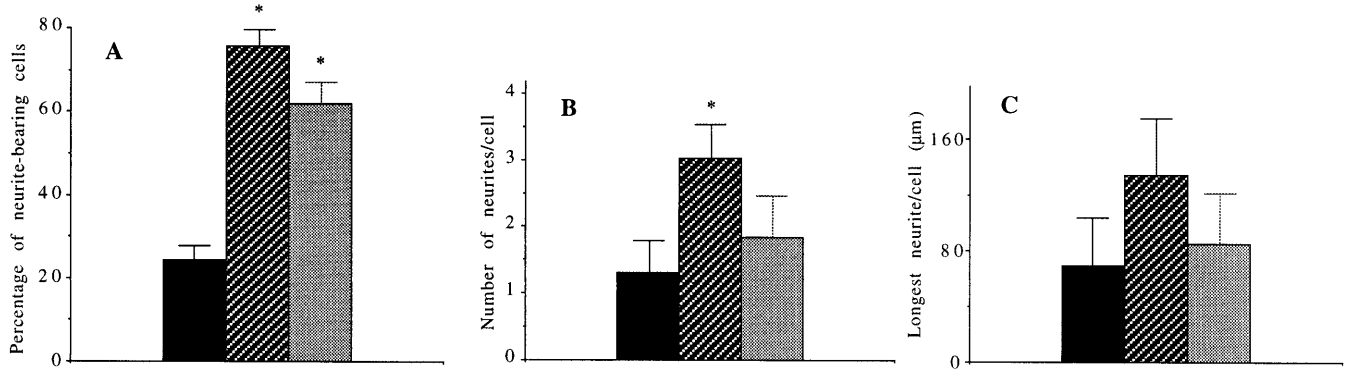


Fig. 4 Effect of pep1 and pep2 on cortical neuronal adhesion. A single cell suspension (1.5×10^5 cells per 16 mm well) was plated onto plastic plates alone (*solid bar*), or plates coated with pep1 (*broadly striped bar*), pep2 (*shaded bar*), or BSA (*narrowly striped bar*). No significant difference was observed in the number of adhering cells between control (*solid bar*) and peptide-treated cultures 1 h after cell seeding. Values represent the mean±SEM of duplicate cultures

Fig. 5A–C Effect of pep1 and pep2 on neurite outgrowth in low-density cortical neuronal cultures. Cells were grown for 4 days in the absence (*solid bar*) or presence of pep1 (*broadly striped bar*) or pep2 (*shaded bar*). The percentage of neurons bearing neurites (**A**), the average number of neurites per cell (**B**), and the average length of the longest neurite per cell (**C**) are shown. Values are the mean±SEM (n=3). *P<0.0001 vs controls (ANOVA)



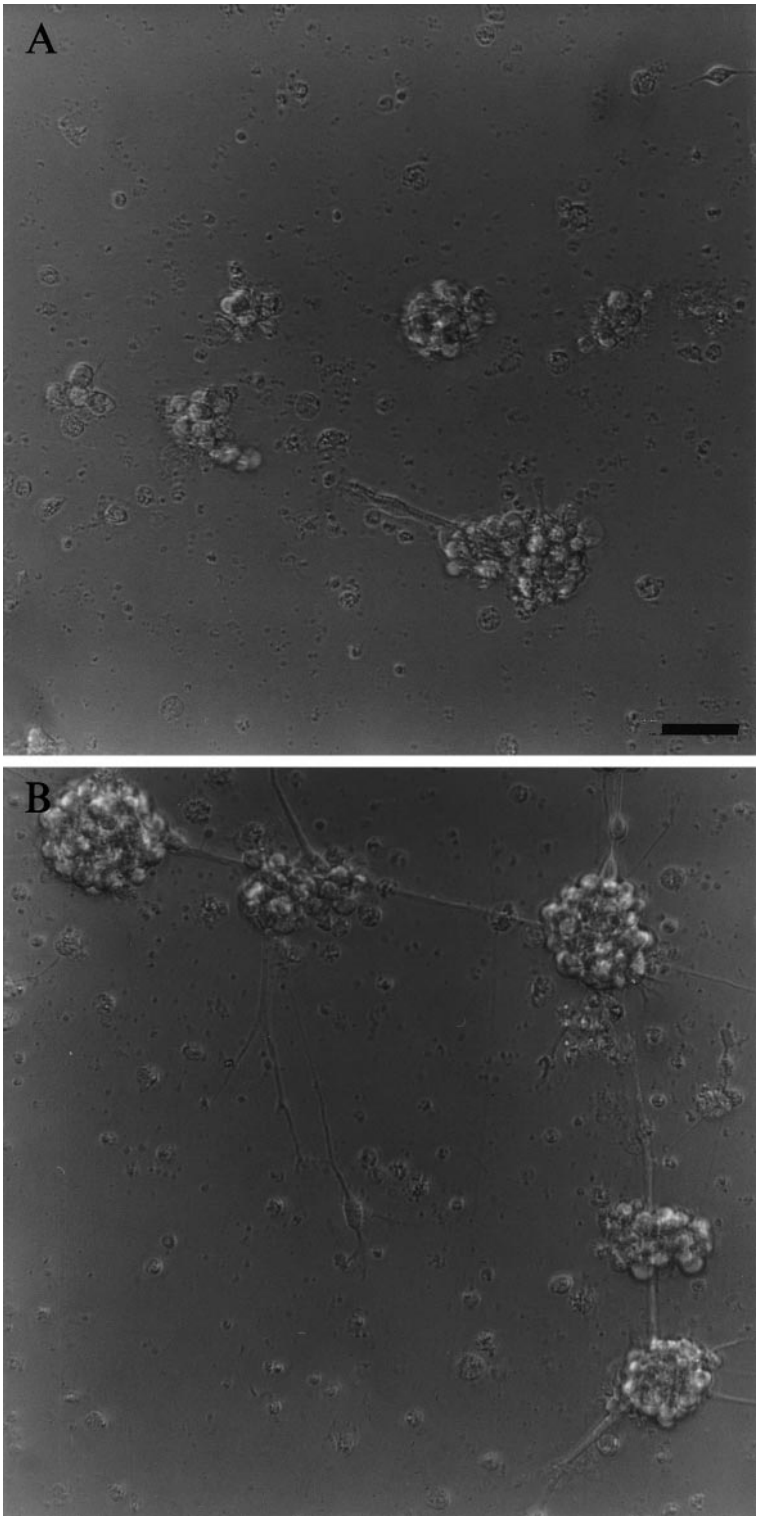


Fig. 6A, B Effect of pep1 on the morphology of cortical neurons grown for 5 days on thrombospondin-coated plates. Phase-contrast micrographs. **A** Control culture with neuronal aggregates exhibiting poor neuritic outgrowth. **B** In the presence of pep1, the aggregates are interconnected by bundles of neuritic processes. *Bar* 50 µm

Pep2: WSPCSVSCG. Pep2 tested at the same concentration as pep1 was less efficient; aggregate detachment was observed with time in culture, in contrast to pep1-treated cultures, and neuritic outgrowth was poorly developed (Fig. 2C vs Fig 2B). Morphometric analysis revealed that the number of neuronal aggregates increased 5-fold from control to pep2-treated cultures (4.33±0.47 to 21.67±4.19) instead of 9.3-fold to pep1-treated cultures (Fig. 3A). For the number of neurites per aggregate (Fig. 3B), a 1.6-fold decrease was observed from pep2 vs pep1-treated cultures (3.90±1.0 between pep2 and controls; 6.18±1.01 between pep1 and controls), whereas the length of the longest neurite per aggregate was unaffected (Fig. 3C).

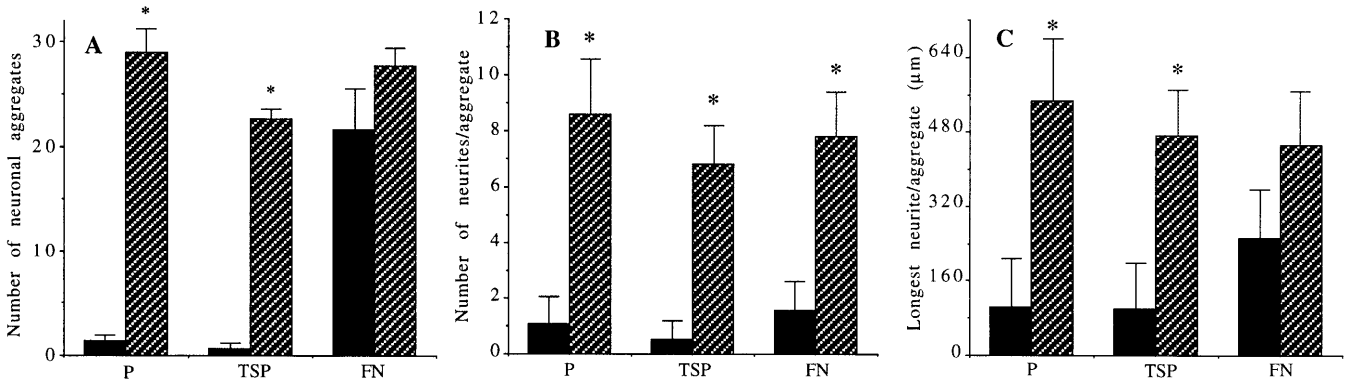


Fig. 7A–C Quantitative analysis of the effect of pep1 on cortical neuronal cell adhesion and neuritic outgrowth in the presence of ECM proteins. Cells were plated on plastic plates alone (*P*), on thrombospondin-coated plates (*TSP*), or on fibronectin-coated plates (*FN*). Cells were maintained without (*solid bar*) or with pep1 (*broadly striped bar*) for 5 days. The number of neuronal aggregates (**A**), the average number of neurites per aggregate (**B**), and the average length of the longest neurite per aggregate (**C**) are shown. Values are the mean±SEM (n=3). *P<0.0001 vs controls (ANOVA)

Pep3: RKAR. No effect was detected on either cell adhesion or neurite outgrowth in the presence of pep3.

LDLr type A-derived peptide

Pep4: DCKDGSDE. No effect was obtained with pep4 spotted at a single dose from 25–200 µg per spot (data not shown).

Effect of TSR-derived peptides under various experimental conditions

Cell-to-substrate adhesion

We looked for a relationship between the ability of pep1 and pep2 to promote neuronal adhesion and the ability to increase cell-to-substratum interactions during the first hour post-plating. As shown in Fig. 4, no difference in the number of adhering cells was detected between control and peptide-treated cultures. On the other hand, BSA was poorly adhesive.

Low-density cultures

To determine whether the neurite outgrowth promoting activity of pep1 and pep2 depended on neuronal aggregation, we cultivated the cells at low density to avoid aggregate formation. As shown in Fig. 5, in the absence of aggregation, both peptides significantly increased the percentage of neuronal cells bearing neurites. In controls, only 24.4% of the adherent cells exhibited neurites after 4 days in culture, whereas a 3-fold and a 2.5-fold increase occurred in the presence of pep1 and pep2, respectively. In addition, a significant increase in both the number of neurites per cell (2.3-fold) and the length of the longest neurite (1.9-fold) was observed in the presence of pep1, but not with pep2.

Different substrates

Since all the above experiments were performed on plastic substrate, we investigated whether the more active peptide, namely pep1, could affect neuronal behavior in the presence of ECM proteins in the same way. In the presence of thrombospondin, pep1 also increased neuritic outgrowth (compare Fig. 6A and 6B). As shown in Fig. 7, the adhesion and thus the number of neuronal aggregates in pep1-treated cultures increased on thrombospondin, compared with control cultures. In the presence of fibronectin alone, neuronal cells adhered more efficiently; this meant that there was no measured difference between pep1-treated and untreated cultures on cell aggregation. However, pep1 significantly increased the number of neurites per aggregate in both thrombospondin- and fibronectin-coated wells compared with controls (13.6-fold and 5-fold for thrombospondin and fibronectin, respectively) and increased the length of the longest neurite per aggregate (4.72-fold and 1.8-fold, respectively).

Presence of heparin

To determine whether the WSXWS sequence in pep1 could interact with proteoglycans, we tested various concentrations of heparin in the presence of pep1 coated onto plastic wells (Fig. 8). In the presence of heparin alone, cortical neuronal cells adhered as poorly as previously shown on the plastic substrate. In contrast, cells seeded onto pep1 in wells in which heparin was added at 40 µg/ml adhered, aggregated with time in culture, and did not display any detachment from the substrate after 5 days in culture. In addition, neuronal aggregates were interconnected by neuritic processes. Quantitative analysis revealed a significant increase in both the number of neurites per aggregate (4-fold) and the length of the longest neurite per aggregate (2.5-fold) from cultures incubated

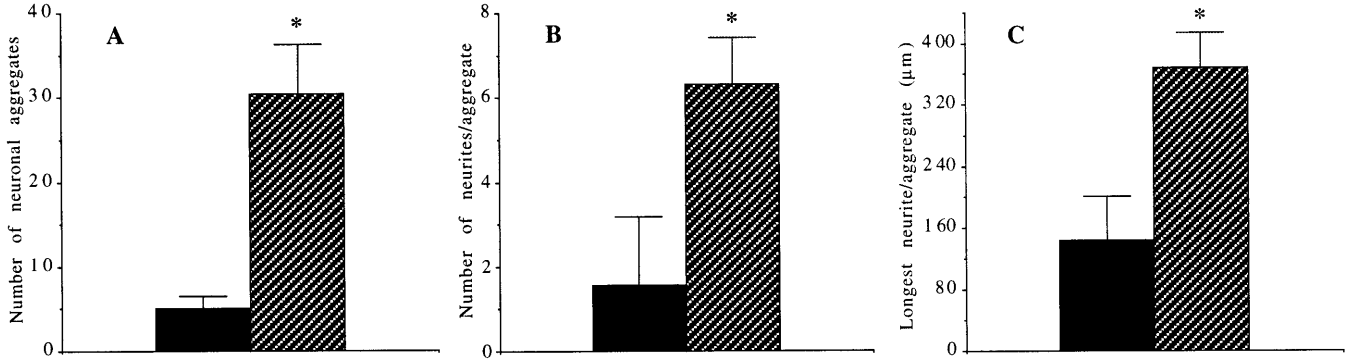


Fig. 8A–C Quantitative analysis of the effect of pep1 on cortical neuronal cell adhesion and neurite outgrowth in the presence of soluble heparin. Cells were grown for 5 days in the presence of 40 μg/ml heparin alone (*solid bar*), or in the presence of both heparin and pep1 (*broadly striped bar*). The number of neuronal aggregates (**A**), the average number of neurites per aggregate (**B**), and the average length of the longest neurite per aggregate (**C**) are shown. Values are the mean±SEM (n=3). *P<0.0001 vs controls (ANOVA)

in the presence of heparin alone compared with those incubated with both heparin and pep1. No inhibition of pep1-mediated cell adhesion was observed in the presence of a higher heparin concentration (80 μg/ml; data not shown). Preincubation of pep1 substrate with 40 μg/ml heparin abolished neither attachment nor neuritic outgrowth, indicating that heparin binding to pep1 was unlikely. As for experiments with soluble heparin, morphometric analysis revealed an increase in both the number of neurites per aggregate (2.4-fold) and the length of the longest neurite per aggregate (2.2-fold) compared with substrate-coated heparin alone (data not shown).

Soluble form

We analyzed whether the peptides could influence the development of cortical neurons in culture, when presented to the cells in a soluble form. When peptides were added at concentrations up to 500 μg/ml, no effect was obtained on either neuronal adhesion or morphology compared with untreated cells seeded on the plastic substrate (data not shown).

Effect of pep1 on spinal-cord neurons

No difference in the number of adhering cells between control and pep1-treated cultures was recorded 1 h after plating (data not shown). The morphology of cultured neurons was different according to the presence or absence of pep1 (compare Fig. 9A, B with Fig. 9C, D). When spinal-cord cells were plated in the presence of pep1-coated substrate, neurons remained evenly distributed for at least one week in vitro. Neurons displayed prominent neuritic outgrowths forming a network of processes with no obvious fasciculation of neurites. In contrast, neuronal cells in controls generally formed small aggregates interconnected via long processes. Neurites grown from the aggregates joined to form relatively straight fascicles along which many single bi- or tripolar neurons could be seen. A quantitative assessment of both neuronal aggregation and neurite growth is shown in Fig. 10. No difference in neuronal morphology was observed between control and pep1 S-treated cultures. In addition, pep2 when tested under the same conditions and at the same concentration as pep1 had no effect on spinal-cord neuronal morphology.

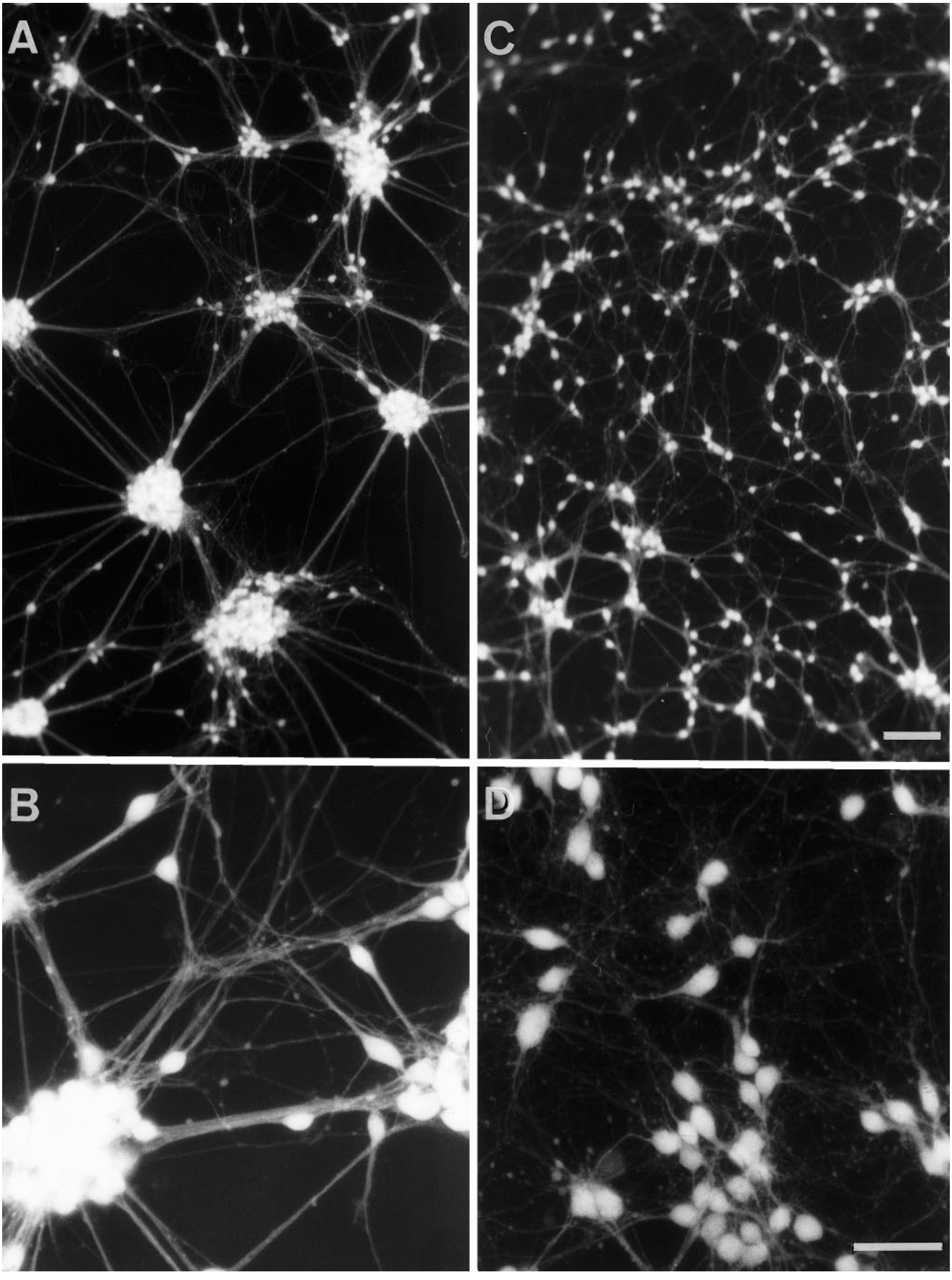
Discussion

We show that two peptides derived from two TSR sequences of SCO-spondin promote the adhesion and neuritic outgrowth of embryonic chick cortical neurons cultivated in serum-free medium. One of these peptides (pep1) is more potent than the other (pep2). The biological effects observed with pep1 are specific, since the scrambled sequence (pep1 S) has no activity under the same conditions. In addition, only pep1 displays different biological activities depending on whether the cells are of cortical or spinal-cord origin.

Ability of TSR-derived peptides to promote adhesion and neuritic outgrowth

Previous studies have demonstrated the ability of thrombospondins to promote adhesion and process outgrowth from central and peripheral neurons (Neugebauer at al. 1991; O'Shea et al. 1991; Osterhout et al. 1992; De Freitas et al. 1995), but the exact mechanisms involved in these processes have not yet been clearly defined. Nevertheless, the neurite-promoting activity has been localized within the region that includes the TSRs (Osterhout et al.

Fig. 9A–D Influence of pep1 on spinal-cord neuronal morphology. Anti-68-kDa neurofilament protein immunofluorescence of neurons grown for 5 days in the absence of any substratum. **A**, **B** Control culture with neuronal aggregates interconnected by bundles of neurites. **C**, **D** In the presence of pep1, spinal-cord neurons remain evenly distributed, and neuritic outgrowth constitutes a dense network. **A**, **C** *Bar* 100 μm. **B**, **D** *Bar* 50 μm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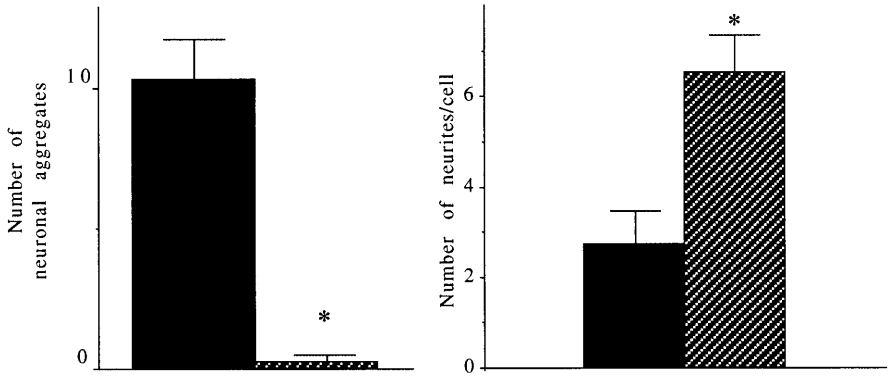

Fig. 10A, B Quantitative analysis of the effect of pep1 on spinal-cord neurons. Cells were plated for 7 days in the absence (*solid bar*) or presence of pep1 (*broadly striped bar*). The number of neuronal aggregates (**A**) and the average number of neurites per cell (**B**) are shown. Values are the mean±SEM (n=2). *P<0.0001 vs controls (ANOVA)

1992; De Freitas et al. 1995). Our results convincingly show that an amino-acid sequence present within a TSR of SCO-spondin is able to modulate both adhesion and neuritic outgrowth from chick CNS neurons.

Some TSRs from identified proteins including thrombospondins contain the amino acid sequence VTCG, which promotes cell adhesion in various biological systems (Prater et al. 1991). Pep2 contains a sequence (VSCG) that displays a single conservative substitution, but this sequence has been shown to be functionally equivalent to the VTCG sequence in properdin (Prater et al. 1991). Although the sequence of pep1 differs from the VTCG consensus domain by two amino acids (RSCG), it is more potent in mediating cortical cell adhesion and neuritic outgrowth than the pep2 sequence.

Thus, the presence of other or additional amino acids in pep1 may increase pep1-mediated cell adhesion and/or neurite outgrowth compared with the less potent pep2; pep1 contains a WSXWS sequence, which is absent from pep2 and which is a site of protein-protein interaction in other biological systems. This motif is also found in the variant fibronectin type III repeat of receptors for several growth and differentiation factors (Bazan 1990; Patthy 1990; Davis et al. 1991), and a mutation of this motif in the interleukin 2 receptor has been shown to block transmembrane signaling (Miyazaki et al. 1991).

The only contribution showing an involvement of peptides with the WSXWS sequence in cell adhesion comes from Guo et al. (1992). These authors have reported that peptides from the TSRs of human thrombospondin containing this consensus sequence promote melanoma cell adhesion when immobilized on plastic. Furthermore, they have defined a distinct class of heparin-binding peptides that lack the currently known heparin-binding consensus sequences (the BBXB domain, where B is a basic amino acid; Cardin and Weintraub 1989).

In addition, the WSPWS sequence has been reported to play a role in neuritic outgrowth (Osterhout et al. 1993) via a heparin-binding moiety, since the promotion of neuritic outgrowth can be inhibited by soluble heparin. Our data, however, do not show any heparin-mediated inhibition on cortical cell adhesion or on neurite extension in the presence of pep1. Thus, the mechanism of pep1 activity remains unclear, but the involvement of heparin-binding molecules of the neuronal cell surface appears unlikely. Nevertheless, recent studies have identified various types of proteoglycans on developing neurons (Margolis and Grumet 1996); these proteoglycans are involved in neuronal development and could function as receptors of SCO-spondin. According to Neugebauer et al. (1991), integrins, which are widely involved in the recognition of ECM components, may function as thrombospondin receptors mediating neurite outgrowth of retinal neurons. Moreover, the integrin $\alpha_3\beta_1$ recognizes the TSRs and mediates neurite outgrowth of rat sympathetic neurons on purified thrombospondin (De Freitas et al. 1995). By analogy, integrins are candidates for a certain class of SCO-spondin receptor, but exactly which amino acids of the TSRs are involved in this response has yet to be determined.

The exact mechanism by which pep1 interacts with neuritic outgrowth remains to be demonstrated, but we emphasize that this activity is highly specific, since it does not appear when using the pep1 S sequence. In addition, ECM substrates, which exhibit some homologies with the pep1 sequence, do not significantly modify the neuronal response to this peptide.

Pep 3 (RKAR), a BBXB sequence that is known to bind proteoglycans in thrombospondin (Adams et al. 1995), has no effect on either cortical neuronal adhesion or neurite outgrowth. Similarly, O'Shea et al. (1991) have shown the inability of a thrombospondin 25-kDa fragment containing the amino-terminal heparin-binding domain to promote either central or peripheral neuronal adhesion or neuritic outgrowth. In addition, a sequence containing both BBXB and VTCG motifs can be inactive with respect to binding heparin (Guo et al. 1992). Even so, under our cell culture conditions, the RKAR motif is not involved in the cell adhesion process.

Concerning the LDLr type A domains, no effect has been detected on cortical neuronal cells by using peptides with highly conserved amino acids of this motif. Thus, in our cell system, this domain is probably not involved in cell adhesion or neurite outgrowth. However, such domains are also present in proteoglycans (Kallunki and Tryggvason 1992) or in morphoregulatory proteins resembling ECM proteins, e.g., the *Drosophila* nudel protein (Hong and Hashimoto 1995). Whether these domains occurring in SCO-spondin are involved in binding to lipoproteins or to some other related molecules requires fur-

ther study. It is interesting to note that Godyna et al. (1995) have identified members of the LDLr family as receptors for thrombospondin in smooth muscle cells, endothelial cells, and fibroblasts.

Putative mechanism(s) of pep1 activity

Adhesion process

Several investigations have demonstrated the relationship between repulsive mechanisms and the cell aggregation process in dissociated central or peripheral neuron cultures. For instance, the chick neural ECM protein restrictin allows retinal cells to adhere and aggregate but not to extend neurite outgrowth (Rathjen et al. 1991). Likewise, embryonic chick forebrain neurons form aggregates without extending processes when plated on GP55, a protein isolated from adult chick brain (Clark and Moss 1994). In several other studies, where CNS neurons have been plated on a substrate containing repulsive molecules, they do not even adhere (Faissner and Kruse 1990; Maeda and Noda 1996). Our results, however, show that pep1 is not an anti-adhesive substrate, as it promotes enhanced neuritic outgrowth. In addition, when pep1 is coated onto fibronectin or thrombospondin substrates, numerous processes extend from the aggregates, suggesting that the effect of pep1 is mainly independent of the type of substrate.

On the other hand, when added in solution, pep1 has no effect on neuronal morphology, suggesting that the conformation of the peptide and its presentation as soluble vs substrate-bound is important for its activity; pep1 is an adhesive substrate that favors neurite development through an increase in cell-to-substrate interactions.

Side-to-side interactions

Our observations demonstrate that pep1 not only promotes neurite outgrowth, but also modifies side-to-side interaction at least in spinal-cord cell cultures; spinal-cord cells no longer aggregate in the presence of pep1 and neurites do not fasciculate, an effect that recalls RF activity on spinal-cord neurons (Monnerie et al. 1997b). In contrast, on cortical neurons, no decrease in side-to-side interaction is observed in the presence of pep1, consistent with the effect of RF on cortical cells (Monnerie et al. 1997a). Pep1, and thus SCO-spondin, might exert opposite effects depending on the cortical or spinal origin of the cells, by acting on different molecules at the neuronal cell surface and/or in relation to their spatio-temporal expression. On the other hand, since neuritic outgrowth is still enhanced in the absence of cell aggregation (cultures at low density), both neuritic outgrowth and aggregative mechanisms can be regarded as being mediated by different sequences of SCO-spondin, as has been demonstrated with tenascin (Husmann et al. 1995). To date, only half of the sequence of SCO-spondin is known (unpublished data); domains other than (or within) the TSRs might be involved in the cell-to-cell adhesion process (Gobron et al. 1996; Orsini et al. 1996).

The results reported here suggest that SCO-spondin displays multifunctional activities during CNS development, involving different domains of the molecule and in relation to the target cell type. In addition, it may be of interest to determine whether domains present in SCO-spondin account for the neuronal survival-promoting activity reported in the presence of RF (Monnerie et al. 1995) and the neuronal anti-aggregative activity observed in the presence of soluble RF-material (Monnerie et al. 1996).

Putative sites of SCO-spondin activity in vivo

SCO-spondin may be widely distributed throughout the CNS, either as a leptomeningeal secretion originating from the basal release of the secretory material (Oksche 1969) or via the RF, leading to the establishment of a gradient of soluble molecules in the developing spinal cord. Depending on the position of the neurons within the gradient and the origin of the cells, from a spinal or cortical source, SCO-spondin could display differential effects on neuronal targets. Using synthetic peptides, we have shown that such variations may exist between cortical and spinal-cord neurons.

Differential adhesive properties are essential during cell migration and differentiation and may influence morphogenetic movements in the developing CNS (Whitesides and LaMantia 1995). Axon elongation and selectivity of growth direction are subjected to regulatory influences (Keynes and Cook 1995). Several glycoproteins with related functions may coexist on identical axons, and differences in the level of expression of specific glycoproteins on distinct axonal subsets could influence the adhesive preferences between cell populations. Both molecular and in vitro studies strengthen the hypothesis that the SCO/RF complex, particularly SCO-spondin, is involved in developmental aspects during CNS formation.

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References

Adams JC, Tucker RP, Lawler J (1995) The thrombospondin gene family. In: Landes RG (ed) Molecular biology intelligence unit. Springer, Berlin Heidelberg New York, pp 1–188

Adams RH, Betz H, Püschel AW (1996) A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis. Mech Dev 57:33–45

Bazan JF (1990) Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934–6938

Bornstein P (1995) Diversity of functions is inherent in matricellular proteins: an appraisal of thrombospondin 1. J Cell Biol 130: 503–506

Bottenstein JE, Sato G (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc Natl Acad Sci USA 76:514–517

Cardin AD, Weintraub HJR (1989) Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis 9:21–32

Clarke GA, Moss DJ (1994) Identification of a novel protein from adult chicken brain that inhibits neurite outgrowth. J Cell Sci 107:3393–3402

Davis S, Aldrich TH, Valenzuela DM, Wong VV, Furth ME, Squinto SP, Yancopoulos GD (1991) The receptor for ciliary neurotrophic factor. Science 253:59–63

DeFreitas MF, Yoshida CK, Frazier WA, Mendrick DL, Kypta RM, Reichardt LF (1995) Identification of integrin $\alpha_3\beta_1$ as a neuronal thrombospondin receptor mediating neurite outgrowth. Neuron 15:333–343

Didier R, Meiniel R, Meiniel A (1992) Monoclonal antibodies as probes for the analysis of the secretory ependymal differentiation in the subcommissural organ of the chick embryo. Dev Neurosci 14:44–52

Faissner A, Kruse J (1990) J1/tenascin is a repulsive substrate for central nervous system neurons. Neuron 5:627–637

Frazier WA (1991) Thrombospondins. Curr Opin Cell Biol 3:792–799

Gobron S, Monnerie H, Meiniel R, Creveaux I, Lehmann W, Lamalle D, Dastugue B, Meiniel A (1996) SCO-spondin: a new member of the thrombospondin family secreted by the subcommissural organ is a candidate in the modulation of neuronal aggregation. J Cell Sci 109:1053–1061

Godyna S, Liau G, Popa I, Stefansson S, Argraves WS (1995) Identification of the low density lipoprotein receptor-related protein (LRP) as an endocytic receptor for thrombospondin-1. J Cell Biol 129:1403–1410

Guo N, Krutzsch HC, Nègre E, Vogel T, Blake DA (1992) Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion. Proc Natl Acad Sci USA 89:3040–3044

Hong CC, Hashimoto C (1995) An unusual mosaic protein with a protease domain, encoded by the *nudel* gene, is involved in defining embryonic dorsoventral polarity in *Drosophila*. Cell 82:785–794

Husmann K, Carbonetto S, Schachner M (1995) Distinct sites on tenascin-C mediate repellent or adhesive interactions with different neuronal cell types. Cell Adhesion Commun 3: 293–310

Kallunki P, Tryggvason K (1992) Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. J Cell Biol 116:559–571

Keynes R, Cook GMW (1995) Axon guidance molecules. Cell 83:161–169

Klar A, Baldassare M, Jessell TM (1992) F-spondin: a gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension. Cell 69:95–110

Lagenaur C, Lemmon V (1987) An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc Natl Acad Sci USA 84:7753–7757

Leung-Hagesteijn C, Spence AM, Stern BD, Zhou Y, Su M, Hedgecock EM, Culotti JG (1992) UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in *C. elegans*. Cell 71:289–299

Maeda N, Noda M (1996) 6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons. Development 122:647–658

Margolis RU, Grumet M (1996) Proteoglycans in neural development. Perspect Dev Neurobiol 3:243–373

Meiniel A, Meiniel R, Didier R, Creveaux I, Gobron S, Monnerie H, Dastugue B (1996) The subcommissural organ and Reissner's fibre complex. Prog Histochem Cytochem 30:1–66

Meiniel R, Molat JL, Duchier-Liris N, Meiniel A (1990) Ontogenesis of the secretory epithelium of the bovine subcommissural organ. A histofluorescence study using lectins and monoclonal antibodies. Dev Brain Res 55:171–180

Miyazaki T, Maruyama M, Yamada G, Hatakeyama M, Taniguchi T (1991) The integrity of the conserved "WS motif" common to IL2 and other cytokine receptors is essential for ligand binding and signal transduction. EMBO J 10:3191–3197

Monnerie H, Boespflug-Tanguy O, Dastugue B, Meiniel A (1995) Reissner's fibre supports the survival of chick cortical neurons in primary mixed cultures. Cell Tissue Res 282:81–91

Monnerie H, Boespflug-Tanguy O, Dastugue B, Meiniel A (1996) Soluble material from Reissner's fibre displays anti-aggregative activity in primary cultures of chick cortical neurons. Dev Brain Res 96:120–129

Monnerie H, Dastugue B, Meiniel A (1997a) Reissner's fibre promotes neuronal aggregation and influences neuritic outgrowth in vitro. Cell Tissue Res 287:285–295

Monnerie H, Dastugue B, Meiniel A (1997b) In vitro differentiation of chick spinal cord neurons in the presence of Reissner's fibre, an ependymal brain secretion. Dev Brain Res 102:167–176

Neugebauer KM, Emmett CJ, Venstrom KA, Reichardt LF (1991) Vitronectin and thrombospondin promote retinal neurite outgrowth: developmental regulation and role of integrins. Neuron 6:345–358

Oksche A (1969) The subcommissural organ. J Neuro Visc Relat [Suppl] IX:111–139

Oksche A, Rodríguez EM, Fernandez-Llebrez P (1993) The subcommissural organ. An ependymal brain gland. Springer, Berlin Heidelberg New York

Olsson R (1993) Reissner's fibre mechanisms: some common denominators. In: Oksche A, Rodríguez EM, Fernandez-Llebrez P (eds) The subcommissural organ. Springer, Berlin Heidelberg New York, pp 33–39

Orsini MJ, Debouck CM, Webb CL, Lysko PG (1996) Extracellular human immunodeficiency virus type 1 Tat protein promotes aggregation and adhesion of cerebellar neurons. J Neurosci 16:2546–2552

O'Shea KS, Liu L-HJ, Dixit VM (1991) Thrombospondin and a 140 kd fragment promote adhesion and neurite outgrowth from embryonic central and peripheral neurons and from PC12 cells. Neuron 7:231–237

Osterhout DJ, Frazier WA, Higgins D (1992) Thrombospondin promotes process outgrowth in neurons from the peripheral and central nervous systems. Dev Biol 150:256–265

Osterhout DJ, Dixit VM, O'Shea KS (1993) The WSPWS motif of thrombospondin promotes neurite outgrowth from embryonic neurons (Abstract). Society for Neuroscience

Patthy L (1990) Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. Cell 61:13–14

Prater CA, Plotkin J, Jaye D, Frazier WA (1991) The properdin-like type I repeats of human thrombospondin contain a cell attachment site. J Cell Biol 112:1031–1040

Rathjen FG, Wolff JM, Chiquet-Ehrismann R (1991) Restrictin: a chick neural extracellular matrix protein involved in cell attachment co-purifies with the cell recognition molecule F11. Development 113:151–164

Schoebitz K, Garrido O, Heinrichs MS, Peer L, Rodríguez EM (1986) Ontogenetical development of the chick and duck subcommissural organ. An immunocytochemical study. Histochemistry 81:31–40

Whitesides III JG, LaMantia AS (1995) Distinct adhesive behaviors of neurons and neural precursor cells during regional differentiation in the mammalian forebrain. Dev Biol 169:229–241