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REGULAR ARTICLE

M. Bamdad · D. Volle · B. Dastugue · A. Meiniel

$\alpha 1\beta 1$-Integrin is an essential signal for neurite outgrowth induced by thrombospondin type 1 repeats of SCO-spondin

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Abstract In the central and peripheral nervous systems a heterogeneous group of proteins constituting the thrombospondin superfamily provides a cue for axonal pathfinding. They either contain or are devoid of the tripeptide RGD, and the sequence(s) and mechanism(s) which trigger in vitro their neurite-promoting activity have remained unclear. In this study, we reconsider the problem of whether sequences present in the thrombospondin type 1 repeats (TSRs), and independent of the well-known RGD-binding site, may activate integrins and account for their neurite-promoting activity. SCO-spondin is a newly identified member of the thrombospondin superfamily, which shows a multidomain organization with a great number of TSR motifs but no RGD sequence. Previous research has implicated oligopeptides derived from SCO-spondin TSRs in in-vitro development of various neuronal cell types. In this study, we investigate whether function-blocking antibodies directed against integrin subunits can block these effects in cell line B104, cloned from a neuroblastoma of the rat central nervous system. By two different approaches: flow cytometry revealing short-term effects and cell cultures revealing long-term effects, we show that: (a) activation of cell metabolism, (b) changes in cell size and structure, and (c) neurite-promoting activity induced by TSR oligopeptides are inhibited by function-blocking antibodies to $\beta 1$-subunit. Using a panel of function-blocking antibodies directed against various integrin α-subunits we show that the $\alpha 1$-subunit might be the partner of the $\beta 1$-subunit in B104 cells. Thus, we demonstrate that an original sequence within a TSR motif from SCO-spondin promotes neurite outgrowth through an intracellular signal driven by integrins, independently of an RGD-binding site.

Keywords SCO-spondin · Thrombospondin type 1 repeats · $\beta 1$-Class integrins · Neurite outgrowth · B104 neuroblastoma cells · Cell culture

M. Bamdad · D. Volle · B. Dastugue · A. Meiniel (✉)
Faculté de Médecine,
UMR INSERM 384 et Laboratoire de Biochimie médicale,
28 place Henri-Dunant, BP 38, 63001,
Clermont-Ferrand Cedex, France
e-mail: annie.meiniel@inserm.u-clermont1.fr
Tel.: +33-4-73178128
Fax: +33-4-73276132

Introduction

SCO-spondin is a newly characterized extracellular protein strongly expressed in the subcommissural organ, a derivative of the ependymal lineage located in the diencephalic roof (Gobron et al. 1996, 2000; cf. Oksche et al. 1993). When secreted at its basal aspect, this protein may be incorporated into the matrix of the posterior commissure, constituting a favorable substrate for pathfinding of commissural axons. In addition, SCO-spondin contributes to the formation of Reissner's fiber, an ontogenically and phylogenically conserved threadlike formation running along the central canal from the edge of the subcommissural organ to the caudal end of the spinal cord (cf. Meiniel et al. 1996, for review). Soluble forms of this protein may be transported via the cerebrospinal fluid and incorporated into various parts of the CNS (cf. Oksche et al. 1993). Thus, this protein may be widely distributed in the vertebrate CNS.

The SCO-spondin gene is expressed early during embryonic development and undergoes a complex regulation leading to the secretion of several SCO-spondin isoforms (Creveaux et al. 1998), which may account, at least partly, for the different forms of the secretion. In addition, the multidomain organization of each isoform may lead to a varying function. The main SCO-spondin isoform, which corresponds to a high molecular mass protein of more than 500 kDa, has a unique mosaic organization of multiple domains which include 26 thrombospondin type 1 repeats (TSRs), nine low density lipoprotein receptor (LDLr) type A domains, two epidermal growth factor (EGF) like domains, and NH_2 and COOH von Willebrand cysteine-rich domains; all these

consensus sequences represent potential sites of protein-protein interaction. Potential binding sites to proteoglycans and growth factors were also identified, but no RGD sequence was found in this protein (Gobron et al. 2000; Meiniel 2001).

Due to the presence of a large number of TSR motifs, SCO-spondin belongs to the thrombospondin superfamily of proteins found in the central (CNS) and peripheral (PNS) nervous systems (Gobron et al. 2000; Adams and Tucker 2000), which includes thrombospondin 1, which is widely distributed in the matrix of the developing nervous system (O'Shea and Dixit 1998), F-spondin 1 and 2 (Klar et al. 1992) and mindin 1 and 2 (Higashijima et al. 1997), secreted at an early stage by the floor-plate cells, and the neuronal transmembrane semaphorins 5A and 5B (Adams et al. 1996). All these molecules are considered positive cues for neurite outgrowth and pathfinding but the exact sequences and mechanisms which trigger this response remain unclear.

Thrombospondin 1 is the most documented member of this thrombospondin superfamily. Its neurite-promoting activity was shown to be mediated by β1-class integrins since antibodies to β1-subunit were able to block the thrombospondin-induced neurite outgrowth of varying neuronal cells (Neugebauer et al. 1991; DeFreitas et al. 1995). In addition, thrombospondin 1 contains an RGD sequence which is well known to activate integrins in response to a variety of extracellular matrix (ECM) molecules such as fibronectin, vitronectin, tenascin, and laminin. Nevertheless, RGD peptides were unable to block the neurite outgrowth induced by thrombospondin 1 (DeFreitas et al. 1995).

We have previously determined sequences in SCO-spondin TSR motifs capable of promoting neurite outgrowth in primary cell cultures of chick cortical and spinal cord neurons (Monnerie et al. 1998) as well as in B104 cell line, cloned from a neuroblastoma of the rat central nervous system (Gobron et al. 2000; El Bitar et al. 2001). Together this has raised the question of whether proteins of the thrombospondin superfamily may contain sequences other than the well-known RGD-binding site, which could activate integrins and promote neurite outgrowth.

In the present study, we demonstrate that the WSG-WSSCSRSCG sequence of a SCO-spondin TSR can promote neurite outgrowth of B104 cells through intracellular signals triggered by an $\alpha1\beta1$-integrin. The possibility that in the other members of the TSR superfamily closely related TSR sequences may, at least partly, account for their neurite-promoting activity through the same ligand/receptor mechanism is also given consideration.

Materials and methods

B104 cell culture

B104 cell line was cloned from a rat neuroblastoma (Schubert et al. 1974). Cells were routinely grown in 75-ml flasks with complete culture medium containing Dulbecco's modified Eagle's medium (DMEM) (Gibco Brl, France) supplemented with 2 mM L-glutamine, penicillin G (50 U/ml), streptomycin sulfate (50 µg/ml), 10% fetal calf serum and 5% horse serum, at 37°C in a humidified incubator in a 90% air and 10% CO_2 atmosphere.

TSR oligopeptide

The activity of the thrombospondin type 1 repeat (TSR) oligopeptide (Syntem, Nimes, France) WSGWSSCSRSCG on neurite outgrowth was previously observed in several cell culture systems (Monnerie et al. 1998; Gobron et al. 2000). This sequence contains the best conserved amino acids of SCO-spondin TSR motifs.

Flow cytometry analysis

Metabolic activity

To analyze rapid intracellular changes after TSR oligopeptide treatment, 5(6)-carboxyfluoresceine diacetate (CFDA) (Sigma, St. Quentin, France) was used to record cell metabolic activity (Petroski and Geller 1994). Cells cultivated for 3 days under the above conditions were washed, trypsinized and centrifuged at 1,400 *t*/min for 7 min. The pellet was removed in serum-free medium and incubated at 37°C.

- For experiments with TSR peptide, 0.75 µg/µl of peptide and 2.5 µM of CFDA were added to 500 µl of serum-free medium containing 300×10^3 cells. B104 cells were incubated at 37°C, and intracellular fluorescence was recorded after 15 min.
- For experiments with monoclonal antibodies (anti-β1-subunit chicken integrin, Sigma, St. Quentin, France; or anti-α3-subunit human integrin, Chemicon, Temecula, CA, USA), 500 µl of serum-free medium containing 300×10^3 cells was treated with 20 µg/ml of function-blocking antibodies (see Testaz et al. 1999 for details) for 15 min at 37°C and then with 0.75 µg/µl of TSR peptide and 2.5 µM of CFDA. Intracellular fluorescence was measured after 15 min at 37°C. In parallel, controls to antibodies were performed in the presence or absence of CFDA dye.
- Three independent experiments were performed.

Cell structure

Cell size and intracellular structural complexity of B104 cells were analyzed under the conditions described above. Each cell population is presented as a pattern of distributed points, one point corresponding to one cell. Size is presented in the *y*-axis and complexity in the *x*-axis. The latter parameter reflects the composition of the intracellular constituents.

Cell metabolic activity and cell structure were analyzed by flow cytometry (FCM), using a Coulter Elite flow cytometer. CFDA was excited at 488 nm with an output power of 15 mW. The fluorescence intensities were obtained using a 525-nm bandpass filter. The results correspond to the mean of 10,000 events, and fluorescence levels were expressed on a logarithmic scale. The mean fluorescence for any given population was expressed in arbitrary fluorescence units (FU). Each experiment was performed in triplicate.

B104 neurite outgrowth in cell culture

For cell culture experiments, non-confluent cells grown for 3 days under the above conditions were plated onto poly-L-lysine-coated 48-well plates at a final density of 1,000 cells/200 µl. Five to 6 h after cell seeding, complete culture medium was replaced by 200 µl serum-free medium per well. These cells were first treated with function-blocking monoclonal antibodies according to the manufacturer's instructions (20 µg/ml of anti-chicken β1-integrin, 20 µg/ml of anti-human α3-integrin, 5 µg/ml of anti-human α1-, α2-, α4-, α5-, α6-, αV-integrins; Chemicon International, Temecula, CA). After 1 h at 37°C, 0.75 µg/µl of TSR oligopeptide was added to the culture medium. For TSR-treated culture without antibodies, 0.75 µg/µl of TSR oligopeptide was directly added to the 200 µl of serum-free medium.

Under these cell culture conditions, neurite extension was studied by recording the number of sproutings and/or the number of neurites and size of the longest neurite per cell. These parameters were examined in bright phase contrast in randomly selected fields using a reticule grid (1×1 cm, subdivided into 100 squares) in the eyepiece of a Leitz DMTL microscope with a magnification of ×125. The size of the longest neurite was determined using the area of one square as reference, defined as one surface unit (1 SU=0.13×0.13 µm with a magnification of ×125). Both parameters were studied in triplicate in each experiment. The number of independent experiments is indicated by *n*.

Statistical analysis

Data were expressed as the means ± SEM of *n* independent experiments. Each experiment was performed in triplicate, and we statistically compared control groups and TSR-treated groups with or without antibody pretreatment with a risk of $P \leq 0.05$. For the number of neurites per cell, the χ^2 independence test was used, and for the size of the longest neurite per cell the parametric Student's test was used. For the experiments in flux cytometry, the quantitative data are relative versus a control which varies for each experiment. Only one experiment is presented in Fig. 2, but we obtained the same pattern threefold.

Results

The SCO-spondin glycoprotein is composed of several types of consensus domains. The active WSGWSSC-SRSCG sequence used in the present study is located within the fourth TSR motif (Fig. 1). This peptide is most efficient on the differentiation process of B104 neuroblastoma cell line. Morphological changes include: (a) cell flattening, (b) increased sprouting and neurite outgrowth, and (c) neuronal aggregation. To determine whether these effects were mediated by an integrin receptor, we used two methods: (a) flow cytometry revealing immediate changes in cell morphology and metabolism, and (b) cell culture revealing with increased time cell flattening and sprouting accompanied by a prominent neurite outgrowth.

β1-Class integrins mediate B104 short-term responses to TSR oligopeptides

Inhibition of TSR-induced cell metabolic activity by antibodies to β1-integrin

Intracellular signals induced by the TSR peptide may be revealed by changes in the metabolic activity of B104 cells through flow cytometry. To determine whether the TSR peptide signal is mediated via an integrin receptor, B104 cells were pretreated with a function-blocking monoclonal antibody to the α3- or β1-integrin subunits, before addition of the TSR peptide. Metabolic activity was studied using CFDA dye (see "Materials and methods"). The intracellular fluorescence was recorded after 15 min of incubation at 37°C by flow cytometry (Fig. 2). The results showed a clear increase in B104 cell metabolic activity in the presence of TSR peptide with 35 FU compared to 16 FU in control. Similarly, in culture pretreated with the blocking antibody to the α3-integrin subunit combined with the TSR peptide, metabolic activity increased to levels comparable to the activity of TSR peptide alone. In contrast, in culture pretreated with the blocking antibody to the β1-integrin subunit and then by the TSR peptide, metabolic activity remained at control level. Cells treated with anti-β1- or anti-α3-subunits alone showed no increase in metabolic activity. Thus only the antibody to the β1-subunit inhibited the metabolic activity induced by TSR oligopeptides.

Inhibition of TSR-induced structural changes by antibodies to β1-integrin

Flow cytometry (FCM) was also performed to compare B104 cell structural changes under the above conditions.

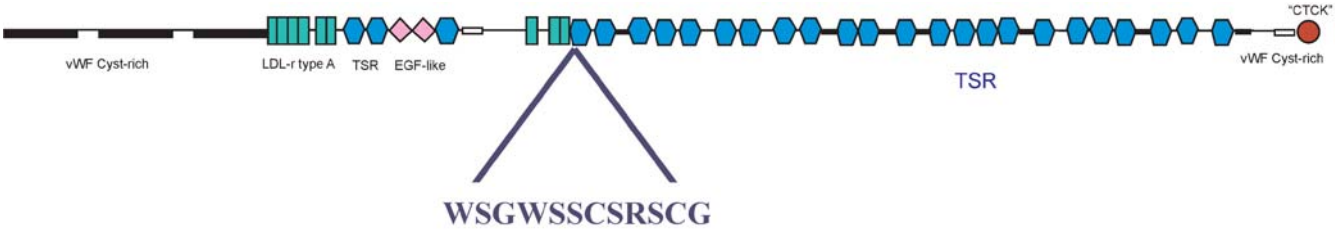

Fig. 1 Schematic representation of SCO-spondin glycoprotein with the position of the WSGWSSCSRSCG oligopeptide within the fourth TSR domain. Multiple domains include 26 thrombospondin type 1 repeats (*TSR*), 9 low-density lipoprotein receptor type A domains (*LDL-r type A*), 2 epidermal growth factor repeats (*EGF-like*), von Willebrand factor cysteine-rich regions (*vWF Cyst-rich*) in the N- and C-terminal regions, and a cysteine knot terminal domain (*CTCK*) in the C-terminal region

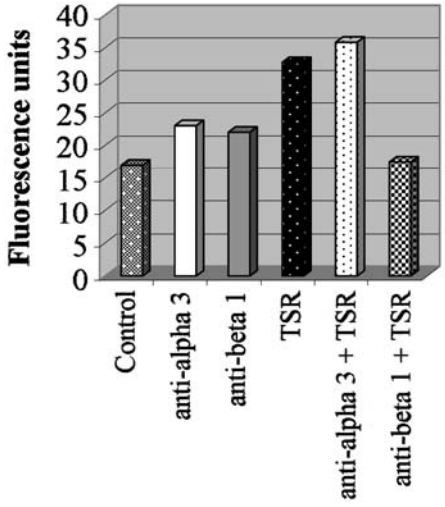

Fig. 2 Analysis of B104 cell metabolic activity by flow cytometry in the presence of TSR peptide with or without pretreatment with blocking antibodies to α3- or β1-integrin. High levels of fluorescence units correspond to an increase in cell metabolic activity (TSR plus anti-α3 + TSR). This pattern corresponds to one experiment and was obtained threefold (see "Materials and methods" for details)

Both parameters—cell size and cell structural complexity—were analyzed since they reflect intracellular changes. The comparison of control cells and TSR peptide-treated cells (Fig. 3B) showed clearly the presence of two different cellular subpopulations with varying cell size and complexity; the control cells were bigger and less complex, while the TSR peptide-treated cells were smaller and more complex. The comparison of B104 cells treated with the TSR peptide in association or without the antibody to the α3-integrin subunit, showed only one cellular population displaying the same cell size and complexity (Fig. 3A). In contrast, B104 cells treated first by the antibody to the β1-subunit and then by the TSR peptide exhibited a pattern of distribution different from that of cells treated exclusively by the TSR peptide. In the presence of the anti-β1-subunit integrin plus TSR peptide, cells resembled the control cells (Fig. 3C). Changes in both FCM parameters may be related to cytoskeletal reorganization upon integrin activation by the

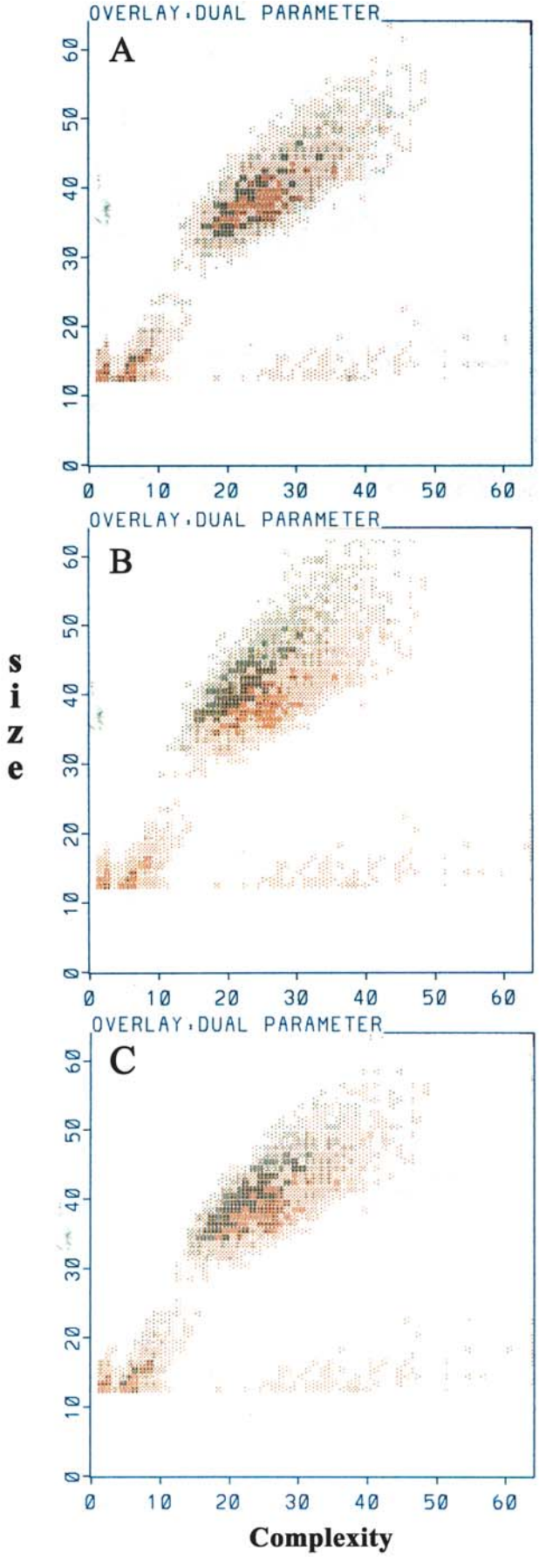

Fig. 3A–C B104 cell size and complexity analyzed by flow cytometry in the presence of TSR peptide with or without pretreatment by blocking antibodies to α3- or β1-integrin. Cell size is expressed by forward scatter = FSC (*y*-axis) and intracellular structure complexity is expressed by side scatter = SSC (*x*-axis). **A** B104 cells treated with TSR peptide (*brown points*) in comparison with cells treated first with a blocking antibody to α3-integrin for 15 min and then with TSR peptide (*black points*). Only one homogeneous cell population with uniform size and complexity was observed. **B** B104 cells treated with TSR peptide (*brown points*) in comparison to control cells (*black points*). Two subpopulations with different size and complexity were clearly detected. **C** B104 cells treated with TSR peptide (*brown points*) in comparison to cells treated first with a blocking antibody to β1-integrin for 15 min and then with TSR peptide (*black points*). Two subpopulations were detected as in **B**

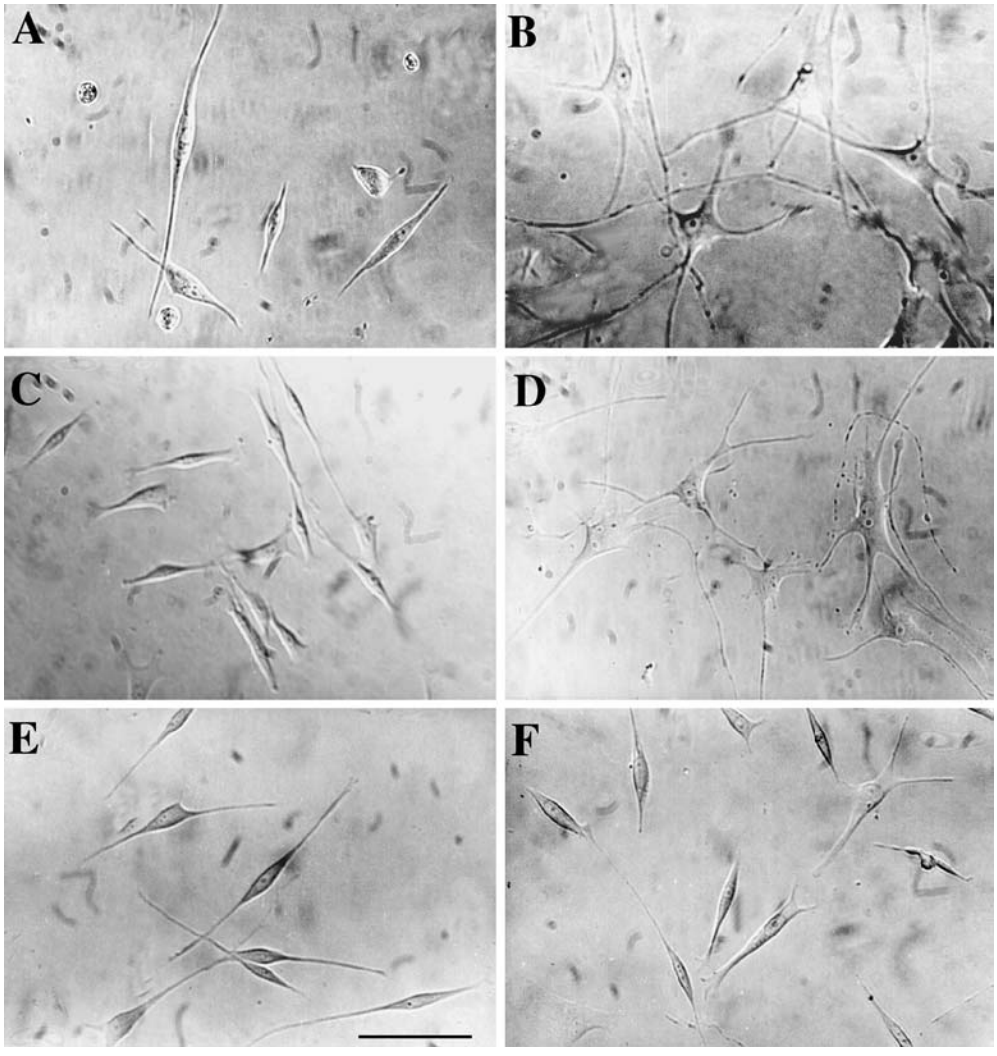

Fig. 4A–F Morphological cell changes of B104 cells over 3 days. **A** Cells in control culture are mainly bipolar. **B** TSR-treated cells with extended neurite outgrowth. **C** B104 cells treated with blocking antibody to α3-integrin resemble controls **(A)**. **D** B104 cells pretreated with blocking antibody to α3-integrin and then treated with 150 µg of TSR peptide resemble TSR-treated cultures **(B)**. **E** B104 cells treated with blocking antibody to β1-integrin do not differ from controls **(A)**. **F** B104 cells pretreated with blocking antibody to β1-integrin and then treated with 150 µg of TSR peptide resemble controls **(A)**. *Bar* 100 µm

TSR oligopeptide. Apparently, a function-blocking antibody to the β1-subunit integrin is capable of inhibiting these changes.

β1-Class integrins mediate B104 long-term responses to TSR oligopeptides

B104 morphological changes

TSR peptide induced with time several morphological modifications in B104 cell culture, including increased (a) cell flattening, (b) cell sprouting, (c) neurite outgrowth, and (d) cell aggregation. To analyze whether all these morphological changes were mediated by an integrin receptor, these events were studied by the use of blocking anti-β1- or blocking anti-α3-integrin subunits.

The change in cell shape was clearly evident from day 1 after TSR treatment. B104 cells were flattened and most of them became tripolar. After 3 days (Fig. 4B), these flattened cells displayed an increased sprouting and neuritic outgrowth. In contrast, in control culture (Fig. 4A), B104 cells retained their characteristic shape and remained refringent and bipolar. B104 cells treated with anti-α3-integrin subunit plus TSR peptide (Fig. 4D) exhibited marked changes similar to B104 cells treated with TSR peptide only. In contrast, after application of anti-β1-integrin subunit plus TSR peptide to the culture (Fig. 4F), almost no morphological cell changes were detected. B104 cells remained bipolar and displayed no marked neuronal processes (Fig. 4A, C, E).

After 4 days in culture (Fig. 5), TSR peptide induced a B104 cell grouping and, later in time, a distinct cell aggregation (Fig. 5B). We analyzed these events in the presence of the blocking antibodies to α3- and β1-integrin. After 4 days, only in B104 cells treated first by anti-α3-integrin and then by TSR peptide (Fig. 5D) were grouping cells detected. In contrast, in cell culture treated first with antibody to β1-integrin and then by TSR peptide (Fig. 5F) no grouping or aggregation was detected during the experiment, resembling controls without exposure to TSR peptide (Fig. 5A) or treated with anti-β1-integrin (Fig. 5E) or anti-α3-integrin alone (Fig. 5C).

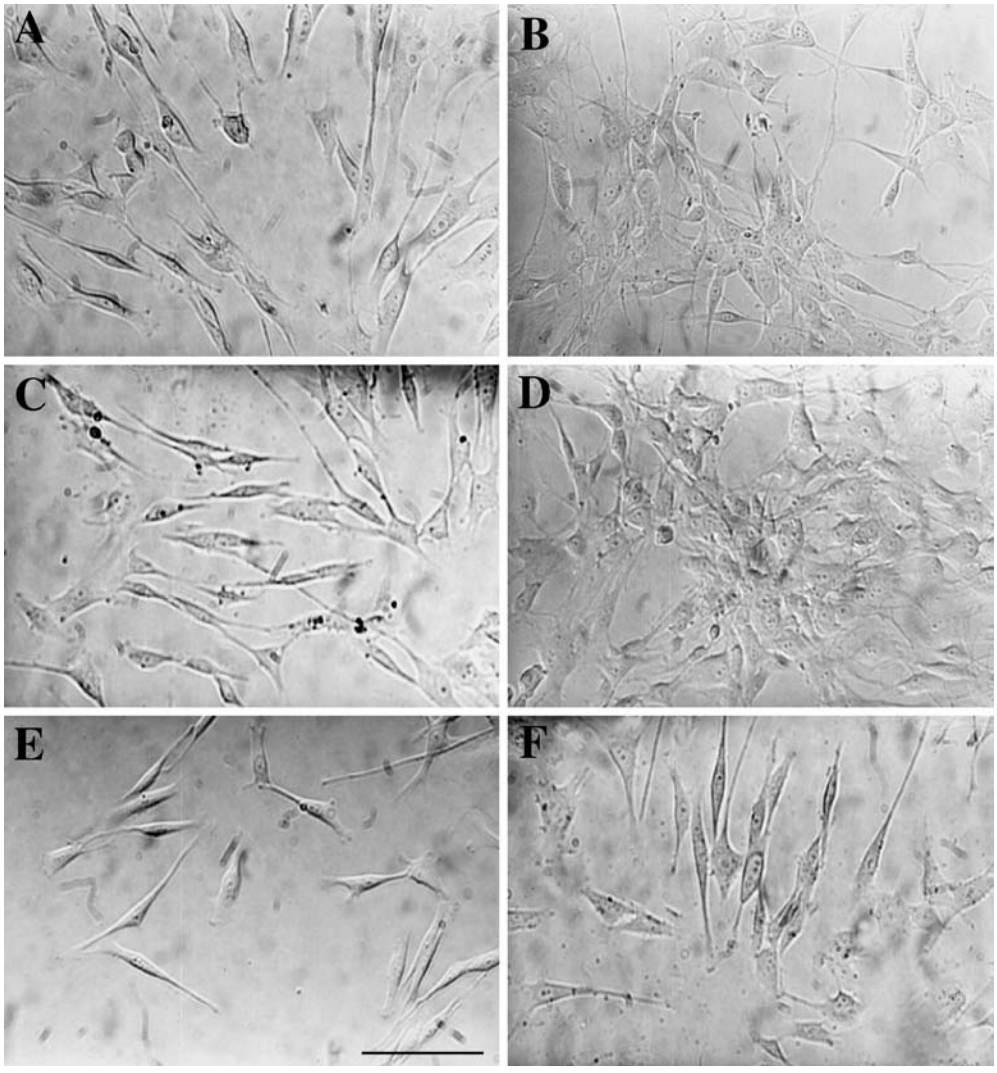

Fig. 5A–F B104-cell grouping at day 4. **A** Scattered cells in control culture. **B** Grouping of TSR treated cells. **C** B104 cells treated with blocking antibody to α3-integrin resemble controls **(A)**. **D** B104 cells pretreated with blocking antibody to α3-integrin and then treated with 150 µg of TSR peptide resemble TSR-treated cells **(B)**. **E** B104 cells treated with blocking antibody to β1-integrin do not differ from controls **(A)**. **F** B104 cells pretreated with blocking antibody to β1-integrin and then treated with 150 µg of TSR peptide resemble controls **(A)**. *Bar* 100 µm

Neurite outgrowth analysis

Changes in cell morphology induced by TSR peptide were always accompanied by an extensive neurite outgrowth. Both the number (n) of neurites and the size of the longest neurite per cell reflected an increased neuronal differentiation induced by this peptide sequence. The kinetics of these two parameters were studied in B104 cell culture, in the presence of anti α3- or anti β1-integrin subunits and TSR peptide up to 48 h (Fig. 6A). The presence of neurites was scored when processes were longer than one cell body diameter. The results were expressed by the percentage of cells exhibiting n neurites. After 12 h, the percentage of cells presenting one or two neurites was higher in TSR peptide-treated culture. Then with increasing time, the percentage of B104 cells endowed with two, three and four neurites increased clearly after treatment with TSR peptide. In contrast, in controls or in anti-α3- or anti-β1-integrin-treated cultures the percentage of cells without a neurite or with only one neurite predominated during the experiment. The percentage of cells with n neurites in culture after treatment with anti-α3-integrin and subsequently TSR peptide resembled that of cultures treated exclusively with TSR peptide. In contrast, in cultures treated with anti-β1-integrin plus TSR peptide the percentage of cells with n neurites resembled that of controls.

The size of the longest neurite per cell was then analyzed up to 48 h (Fig. 6B). In the presence of TSR peptide this parameter increased clearly from 12 h (138.72±19 µm) with a maximum of 250.7±24 µm at 36 h. In regular controls, and in anti-β1-integrin or anti-α3-integrin controls, this parameter remained relatively stable at 96±9, 112±16.5 and 122.72±12 µm at 36 h, respectively. In cultures treated with anti-α3-integrin plus TSR peptide, the size of the longest neurite increased to levels characteristic for TSR-treated cultures, with a maximum of 221.3±27 µm at 36 h. In contrast, in cultures treated with anti-β1-integrin plus TSR peptide, this parameter remained at control level at 98.7±18 µm. Neurite outgrowth induced by TSR peptide in B104 cells

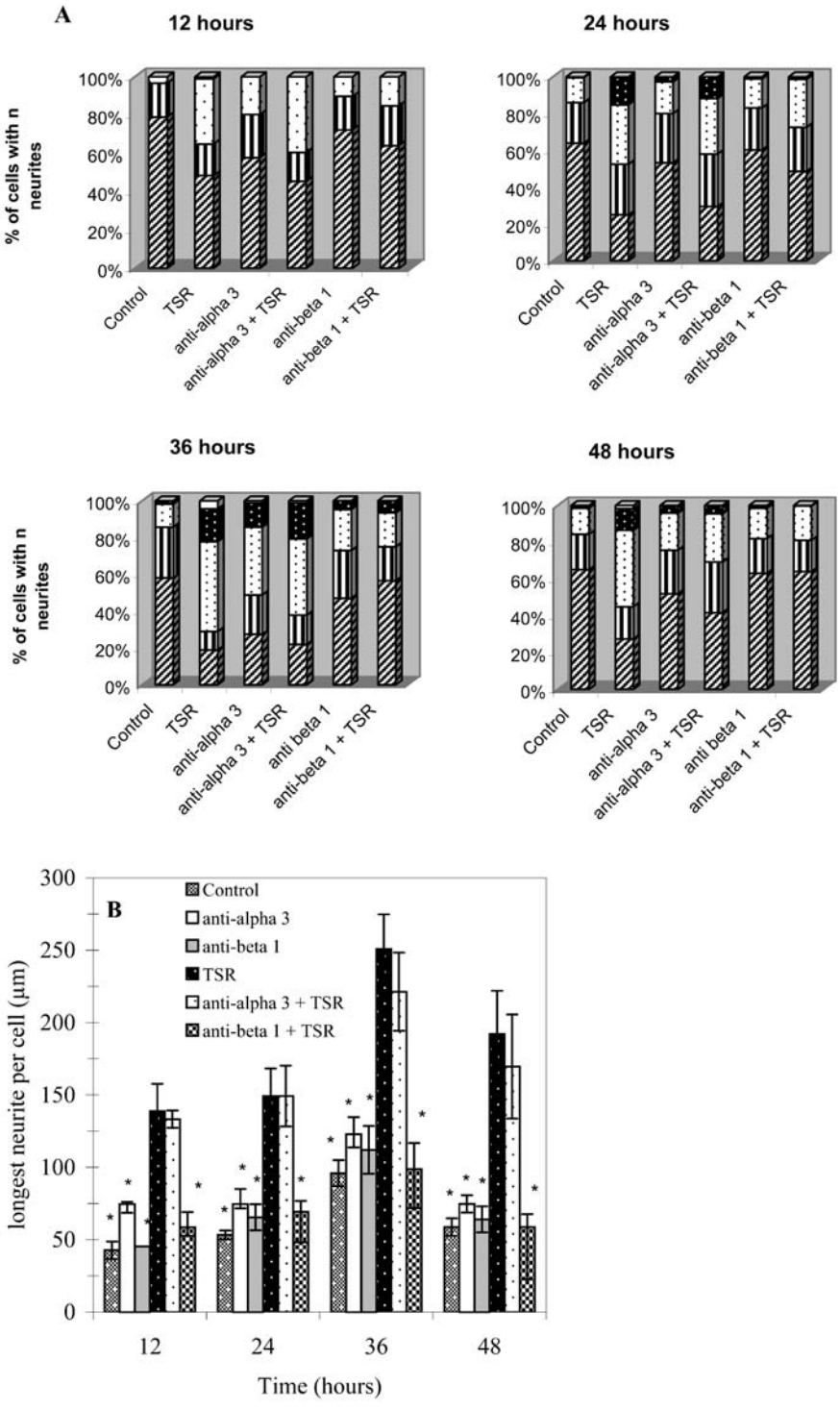

Fig. 6A, B Neurite outgrowth of B104 cells with time in culture, after 12, 24, 36 and 48 h. **A** Percentage of cells with *n* neurites: *diagonal stripes* 0 neurites, *vertical stripes* 1 neurite, *black dots on white* 2 neurites, *white dots on black* 3 neurites, *no hatching* 4 neurites. **B** Size of the longest neurite per cell. Both parameters **A** and **B** increased with TSR peptide or with anti-α3 + TSR peptide. Note the clear inhibition of these parameters with TSR peptide + anti-β1-integrin. Each experiment was performed in triplicate (mean ± SEM; *$P \leq 0.05$ vs TSR or anti-alpha3 + TSR)

was clearly inhibited by the antibody to the β1-integrin subunit.

α1-Integrin is a possible partner of β1-integrin in TSR-mediated long-term responses

In B104 cell cultures, the responses to TSR peptide appeared to be mediated by β1-class integrins. Since α3-subunit was not implicated in TSR peptide response, the role of the α-subunit partner of β1-integrin was then investigated by screening a panel of function-blocking antibodies directed against α1-, α2-, α4-, α5-, α6- and αV-subunits. Under the same conditions as described above, B104 cells were first treated with 5 µM of blocking anti-α-subunit integrin and then with TSR peptide. The number of sproutings and neurites as well as the size of

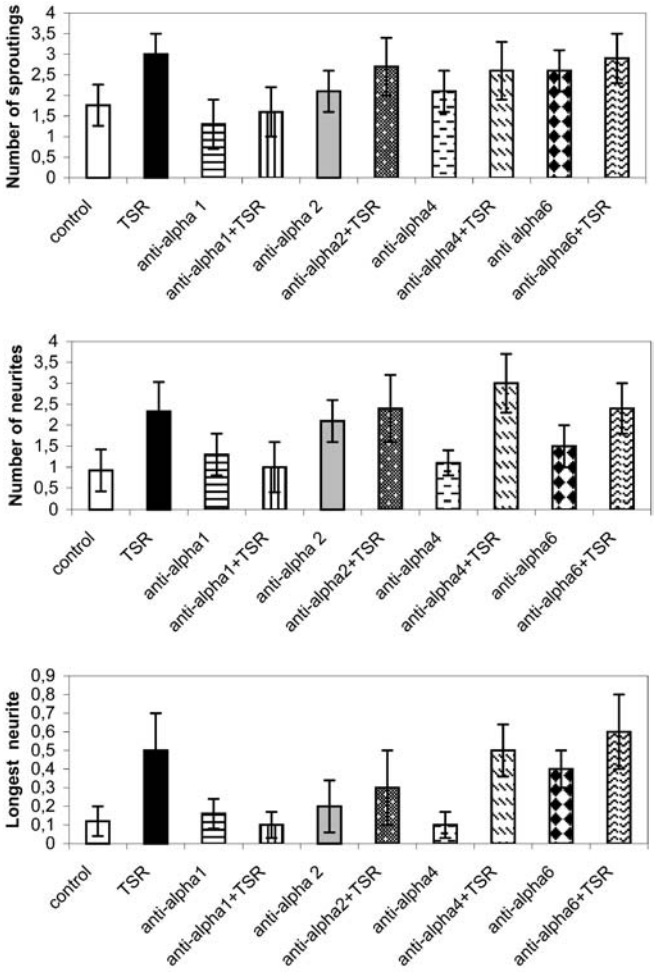

Fig. 7 Sproutings and neuritic outgrowth in B104 cell culture in the presence of blocking antibodies to α1-, α2-, α4- and α6-subunit integrin at day 2. *Top* Number of sproutings; *middle* number of neurites; *bottom* size of the longest neurite per cell. These three parameters were inhibited only with the blocking antibodies to α1-integrin. Each experiment was performed in triplicate (mean ± SEM)

the longest neurite per cell were analyzed in B104 cell culture after 48 h of TSR peptide treatment (Fig. 7).

Only in B104 cell culture treated with antibody to α1-integrin subunit plus TSR peptide did the sprouting (Fig. 7A), number of neurites (Fig. 7B), and size of the longest neurite (Fig. 7C) per cell resemble those of control cells. After application of blocking anti-α2-, -α4-, and -α6-integrin subunits, B104 cell responses were similar to those in the TSR-treated culture.

For cells pretreated with anti-α5- and -αV-subunits, a clear aggregation was detected after 2 days in culture (data not shown).

Discussion

In a B104 cell culture system we show that the neurite-promoting activity and the increased cell-to-cell interaction induced by SCO-spondin TSR oligopeptides can be inhibited by function-blocking antibodies to the β1-integrin subunit. In addition, by flow cytometry we observed an inhibition of the cell metabolism, and changes in cell size and complexity induced by these oligopeptides. Our results provide evidence that β1-integrin signaling can be driven by a novel sequence WSGWSSCSRSCG located within the fourth SCO-spondin TSR, and add to the concept that β-integrin subunit activation might be more complex than previously suspected. Nevertheless, it remains to be determined whether the WSGWSSCSVSCG sequence directly binds to the integrins or to an integrin-associated receptor capable of activating integrins.

Integrins are transmembrane receptors implicated in many functions. They are heterodimeric proteins composed of α- and β-subunits. During embryonic development, their activity is associated with cell migration on a number of ECM proteins (see Perris and Perissinotto 2000 for a review). The adhesion of integrins to their ligands is regulated by conformational changes, intracellular signal transduction pathways and redistribution of integrins on the cell surface associated with a reorganization of the cytoskeleton. Cell adhesivity and locomotory responses to varying ECM molecules require the recruitment of integrins, and the RGD sequence in most ECM proteins has been shown to represent an important functional binding site for integrins in intracellular signaling (Monier-Gavelle and Duband 1997). Locomotory activity of cells on ECM substrates depends on their adhesivity to the various substrates.

Also, directional axonal growth in the course of development depends on the strength of the cell-to-substratum adhesivity (Lochter et al. 1995), in addition to other environmental cues including cell-to-cell interaction and gradients of chemoattractant and chemorepellent molecules (see Cook et al. 1998 for a review). Thus, several ECM molecules implicated in cell migration such as laminin, tenascin, vitronectin, and fibronectin were shown to act on directional axonal pathfinding, involving proteins of the thrombospondin superfamily. For example, thrombospondin 1 acts as a permissive substrate for migration of neural crest cells in the anterior or posterior half of the somite (Tucker et al. 1999). Furthermore, it promotes the migration of cerebellar cells and oligodendrocytes (O'Shea et al. 1990; Scott-Drew and Ffrench-Constant 1997) and also the extension of neurites from several types of neurons, i.e., sensory, sympathetic, spinal cord and retinal nerve cells (O'Shea et al. 1991; Neugebauer et al. 1991; Osterhout et al. 1992; DeFreitas et al. 1995). F-spondin, which is an inhibitory signal for neural crest cell migration (Debby-Brafman et al. 1999), promotes neurite extension (Klar et al. 1992) and peripheral nerve regeneration (Burstyn-Cohen et al. 1998).

Recently several reports have shown an implication of integrins in the process of neurite outgrowth. The activation of β1-class integrins was associated with the neurite-promoting activity on certain ECM proteins; for

example, $\alpha1\beta1$ and $\alpha v\beta1$ of human neuroblastoma TR 14 cells bound to collagens and fibronectin (Carmeliet et al. 1994), $\alpha8\beta1$ of embryonic chick motor and sensory neurons bound to tenascin-C (Varnum-Finney et al. 1995), and $\alpha6\beta1$ associated with L1 and CD9 of neuroblastoma N2A cells were implicated in cell migration and neurite outgrowth on laminin (Schmidt et al. 1996). Moreover, integrin-linked kinase (ILK), a recently identified serine/threonine protein kinase that interacts with the $\beta1$-subunit, was shown to be crucial for neurite outgrowth of mouse neuroblastoma N1E-115 cells seeded on laminin (Ishii et al. 2001). Thus, intracellular signaling cascades that are activated by integrins binding to ECM substrates appear to be essential in the process of neurite extension.

To find out the neurite-promoting domains of ECM proteins, antibodies directed against various regions of the proteins or proteolytic fragments were generated, and a large part of the ECM proteins were defined as containing neurite-promoting activity, for example, the region of tenascin-C containing fibronectin type III repeats 6–8 (Varnum-Finney et al. 1995), the FS1/FS2 domains of F-spondin (Burstyn-Cohen et al. 1998) or the region of thrombospondin-1-containing procollagen-like and TSR domains (Osterhout et al. 1992; DeFreitas et al. 1995). For thrombospondin 1, this region does not contain the RGD-binding site and in addition RGD peptides were unable to inhibit the neurite-promoting activity of thrombospondin (DeFreitas et al. 1995).

Our results convincingly show that the WSGWSSC-SRSCG sequence present within a TSR motif of SCO-spondin promotes neurite outgrowth and cell-to-cell interaction of B104 cells. Furthermore, since antibodies to $\beta1$-integrin can inhibit these biological responses, it can be concluded that the intracellular signaling of $\beta1$-integrin mediates the increased neurite outgrowth and cell aggregation induced by the WSGWSSCSVSCG sequence. Also, the inhibition by these function-blocking antibodies of the immediate changes in B104 cell metabolism, size and complexity induced by the oligopeptide favors rapid intracellular changes following the activation of $\beta1$-integrin by the WSGWSSCSVSCG sequence. Thus, $\beta1$-class integrins can be regarded as a class of receptors involved in the neurite-promoting activity of SCO-spondin TSR. In addition, we show that the same sequence, through the same ligand/receptor activation, can induce different biological responses including neurite outgrowth and cell aggregation. The minimal active sequence and/or the amino acids of this oligopeptide important for its activity remain to be determined. Our results showing an activation of $\beta1$-integrin by the WSGWSSCSVSCG sequence to promote neurite outgrowth of B104 cells are in line with the fact that antibodies to $\alpha3$- and $\beta1$-integrins are capable of inhibiting the neurite outgrowth of retinal and sympathetic neurons on thrombospondin 1 (Neugebauer et al. 1991; DeFreitas et al. 1995).

The mechanisms of integrin β-subunit activation by the α-subunits appear much more complex than previously suspected. Other sequences apart from the well-known RGD-binding site were shown to link integrin α-subunits, for example, the NKDIL and EPDIM sequences from the 2nd and 4th fac-1 domains of TGF-β-induced gene (βig-h3), which activates $\alpha3\beta1$-integrins (Kim et al. 2000), or the $\alpha3$(IV) collagen chain 179–206 peptide, which activates the $\alpha v\beta3$-CD47 complex (Shahan et al. 1999). On the other hand, integrin-associated transmembrane receptors can activate $\beta1$-integrin intracellular signaling, for example, uPAR (urokinase receptor), which associates and binds to αM-subunit through a W4 domain, independently of the I-domain (Simon et al. 2000), or CD47, which binds thrombospondins (Brown and Frazier 2001) or collagens (Shahan et al. 1999). Thus our results add to the concept that integrin β-subunit activation is complex, but it remains to be determined whether the WSGWSSCSVSCG sequence directly binds to integrins or an integrin-associated receptor capable of activating integrins. Since $\alpha3\beta1$ was shown to be involved in the neurite-promoting outgrowth of sympathetic neurons induced by thrombospondin 1 (DeFreitas et al. 1995), we tested the effect of $\alpha3$ function-blocking antibodies in the presence of the SCO-spondin TSR peptide, but no response was observed in our B104 cell culture system. Since anti-$\alpha1$ inhibited the cell flattening, sprouting and neurite extension induced by the TSR peptide, $\alpha1\beta1$ probably mediates B104 response. Nevertheless, it is clear that the repertoire of $\beta1$-integrins capable of linking this TSR sequence may vary regarding the type of neural cells. In addition, the cooperation between several integrins on the same cell type needs to be considered (Testaz and Duband 1999), as well as the presence of a specific integrin-associated receptor (see above). Nevertheless, the TSR motif appears as an important functional domain in the process of neurite extension.

The TSR motif has been recruited in several biological systems to mediate protein-protein interactions. Several binding sites have been identified within the TSR motifs of thrombospondin 1, including the CSVTCG sequence, which activates CD 36/thrombospondin receptor/platelet glycoprotein IV for platelet aggregation (see Adams and Tucker 2000 for a review). In the nervous system, the CSXXCG and WSXWS sequences present within the TSR motifs of thrombospondin 1 have been defined as potent binding sites to neuronal transmembrane proteoglycans, since the activity of peptides comprising these sequences could be inhibited by heparin (see Adams and Tucker 2000; Chen et al. 2000 for reviews). However, we have previously demonstrated on cortical and spinal cord neurons of embryonic chick that the neurite-promoting activity induced by the WSGWSSCSVSCG sequence used in the present work is not inhibited by heparin, suggesting that proteoglycans might not function as the neuronal receptor for this sequence to promote neurite outgrowth (Monnerie et al. 1998). In addition, we have reported that heparin alone can interfere with cell-to-cell interaction and neurite outgrowth (Monnerie et al. 1996).

Thus, our results favor an activation of $\beta1$ intracellular signaling in response to the WSGWSSCSVSCG sequence

and provide a putative mechanism by which ECM proteins of the thrombospondin superfamily could promote neurite outgrowth. However, while SCO-spondin is mainly constituted of TSR motifs, the other relatives of the thrombospondin superfamily show consensus domains not found in SCO-spondin which can modulate the activation of β1-integrins through sequences and mechanisms remaining to be demonstrated. For example, F-spondin 1 and 2 as well as mindin 1 and 2 contain FS1 and FS2 domains, and antibodies raised against these domains were shown to inhibit, at least partly, the neurite-promoting activity of F-spondin (Burstyn-Cohen et al. 1998). Also, F-spondin 1 and 2 show a reelin domain while the semaphorins F and G contain a semaphorin domain, in addition to TSR motifs (Klar et al. 1992; Adams et al. 1996). For SCO-spondin the activity of TSR oligopeptides on neurite outgrowth and cell-to-cell interactions correlates well with that of Reissner's fiber, which contains a high concentration of full-length SCO-spondin molecules (Gobron et al. 2000).

We have characterized a short sequence within a TSR motif of SCO-spondin which enhances neurite outgrowth and cell-to-cell interaction through intracellular signaling triggered by integrins. Thus, β1-integrins can be regarded as a class of receptors involved in the neurite-promoting activity of SCO-spondin. Considering the large number of TSR motifs that contain the WSXWSXCSXXCG consensus, this protein might be highly potent in vivo due to its influence on axonal pathfinding in the area of the posterior commissure (see Gobron et al. 2000 for details). In addition, we show that the same sequence, through the same ligand/receptor activation, can induce different biological responses such as neurite outgrowth and cell aggregation.

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