F-spondin and mindin: two structurally and functionally related genes expressed in the hippocampus that promote outgrowth of embryonic hippocampal neurons

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SUMMARY

Extracellular matrix (ECM) proteins play an important role in early cortical development, specifically in the formation of neural connections and in controlling the cyto-architecture of the central nervous system. F-spondin and Mindin are a family of matrix-attached adhesion molecules that share structural similarities and overlapping domains of expression. Genes for both proteins contain a thrombospondin type I repeat(s) at the C terminus and an FS1-FS2 (spondin) domain. Both the vertebrate F-spondin and the zebrafish mindins are expressed on the embryonic floor plate. In the current study we have cloned the rat homologue of mindin and studied its expression and activity together with F-spondin in the developing rodent brain. The two genes are abundantly expressed in the developing hippocampus. In vitro studies indicate that both F-spondin and Mindin promote adhesion and outgrowth of hippocampal embryonic neurons. We have also demonstrated that the two proteins bind to a putative receptor(s) expressed on both hippocampal and sensory neurons.

Key words: Hippocampus, Neurite outgrowth, Adhesion, Rat, F-spondin, Mindin

INTRODUCTION

Two of the most remarkable features of the developing nervous system are the ability of neuronal cells to migrate to distant locations and the ability of axons to specifically innervate target neurons. The involvement of transient cell populations, including both neurons and glia, in the formation of neural connections and in organizing the structure of the central nervous system, has been clearly demonstrated (Tessier-Lavigne and Goodman, 1996). Recent studies have shown that adhesion molecules can guide axons by either promoting or inhibiting outgrowth. These outgrowth modulators can act as short-range membrane-attached proteins, secreted extracellular matrix proteins or long-range diffusible molecules (Tessier-Lavigne and Goodman, 1996). It is the relative balance between attractive and repulsive forces that regulates the directionality of axonal outgrowth during development.

Recent studies have shown that several extracellular matrix (ECM) proteins, such as Reelin (D’Arcangelo et al., 1995; Ogawa et al., 1995), Tenascin (Faissner, 1997), Fibronectin, Chondroitin sulfate proteoglycan and Heparin sulfate proteoglycan (Pearlman and Sheppard, 1996), play a role in early cortical development. We have previously isolated a floor plate gene, F-spondin, which encodes an extracellular matrix (ECM) protein of 807 amino acids with adhesive properties (Klar et al., 1992a,b; Ruiz-i-Altaba et al., 1993). The carboxyl half of the protein (amino acids 440-807) contains six thrombospondin-type 1 repeat(s) in the C terminus and an FS1-FS2 (spondin) domain. Both the vertebrate F-spondin and the zebrafish mindins are expressed on the embryonic floor plate. In the current study we have cloned the rat homologue of mindin and studied its expression and activity together with F-spondin in the developing rodent brain. The two genes are abundantly expressed in the developing hippocampus. In vitro studies indicate that both F-spondin and Mindin promote adhesion and outgrowth of hippocampal embryonic neurons. We have also demonstrated that the two proteins bind to a putative receptor(s) expressed on both hippocampal and sensory neurons.

Key words: Hippocampus, Neurite outgrowth, Adhesion, Rat, F-spondin, Mindin
(F-spondin containing six, and Mindin only one). Thus, vertebrate Mindins and F-spondin represent a family of proteins that share structural and biochemical similarities.

Recombinant F-spondin promotes neural cell adhesion and neurite extension of DRG (Burstyn-Cohen et al., 1998; Klar et al., 1992a) and spinal cord neurons (Burstyn-Cohen et al., 1999) in vitro. We have shown that F-spondin is present in peripheral nerves during embryonic development, but by birth it is present only in diminished amounts. Axotomy of the adult sciatic nerve causes a massive upregulation of both F-spondin mRNA and protein distal to the lesion, where it is able to promote neurite outgrowth of sensory neurons (Burstyn-Cohen et al., 1998). Perturbation of F-spondin protein levels in the spinal cord in vivo results in pathfinding errors of the commissural axons on the floor plate. This suggests that F-spondin is required for accurate pathfinding of commissural axons at the floor plate (Burstyn-Cohen et al., 1999).

In this study we have cloned the rat mindin gene. Unlike the zebrafish genes, which are mainly expressed in the floor plate, the rat gene is expressed in other brain areas including the hippocampus, a region in which Reelin plays a role in both migration and axonal growth (Del Rio et al., 1997; Nakajima et al., 1997; Ogawa et al., 1995). We have also shown that the expression patterns of F-spondin and mindin overlap in the developing and adult rodent brain, indicating that the two proteins share structural similarities and also comparable expression patterns. Furthermore, we demonstrate that both F-spondin and Mindin bind to a putative receptor(s) expressed in hippocampal neurons, promoting neural cell adhesion and neurite outgrowth.

**MATERIALS AND METHODS**

**Isolation and characterization of rat mindin**

Rat mindin was amplified from rat genomic DNA by the polymerase chain reaction (PCR) using degenerate primers corresponding to two regions of conserved amino acid sequence: PSPDW (ATY GTN CCN WSN CCN GAY TGG) and DAGTD (GAY GCN GGN ACS GAY ANN). The sequence 3’ of the PCR fragment was amplified using the RACE kit (Boehringer). The primers used for the 3’ RACE were: GCTCTCGAGCTCTGGATGGGAG and GGGTACCTTTTG-TGGGCATCGACGT. The 3’ PCR product was used as a probe to screen a rat brain cDNA library. The mindin cDNA was identified and sequenced.

**DNA constructs**

To generate the Mindin-His expression vector (pSecMindin), a pair of PCR primers, ATTTCTAGTGCAGCTCAGCAGCCAGC and CATCTCGAGGAGACAGCTTATCTGGGGC, were used to generate the full-length mindin gene by PCR. The PCR product was subcloned into the Nhel and XhoI sites of pSecTagB (Invitrogen).

To generate the Min(-T)-His expression vector (pSecMin-T) a pair of PCR primers, ATTTCTAGTGCAGCTCAGCAGCCAGC and CATCTCGAGGAGACAGCTTATCTGGGGC, were used to generate the region corresponding to amino acids 1-275 of the mindin gene by PCR. The PCR fragment was subcloned into the Nhel and XhoI sites of pSecTagB (Invitrogen).

To generate the Mindin-AP expression vector, the alkaline phosphatase gene was excised from the pAPtag1 plasmid and inserted into the blunted XhoI site of pSecMin-T.

To generate the pAP-TS expression vector a pair of PCR primers, GAGAGATGC TCTGGCAACTCCAACCTGGTGTC and AGATCTAGACCTTACCAACTGCCG, were used to generate the region corresponding to amino acids 43-807 of F-spondin by PCR. The PCR fragment was ligated to a HindIII-BglII AP fragment of pAPtag4 plasmid and the ligated product was inserted into the pcDNA3 expression vector (Invitrogen) previously cut with HindIII and XhoI.

To generate the p167 plasmid (for expressing amino acids 45-167 of mindin) a pair of PCR primers, CAGGATCCGGAATTGAGGCCAGACACAGCA and CGAAGCTTGA GCCACACAGCA were used. The PCR fragment was subcloned into the BamHI and HindIII sites of pGEX vector.

To generate the Min(-T)-AP expression vector (pSecMin(-T)-AP), the alkaline phosphatase gene was excised from the pAPtag1 plasmid by cutting with HindIII-HpaI and inserted into the blunted XhoI site of pSecMin-T.

**Production of anti-mindin antibodies**

The p167 was introduced into E. coli. Protein expression was induced by IPTG, and the recombinant protein was purified by absorption onto a column of glutathione-agarose beads (Sigma) according to the manufacturer’s directions. The purified protein was injected into rabbits (250 μg protein/injection in adjuvant; total of three injections), and the serum was tested for immunoreactivity by western blotting.

**In situ hybridization and immunocytochemistry**

Mouse embryos from stage E12, E14, E16 and E18, mice from postnatal stages P0, P5, P10, P15, P21, adult mice, and rats from postnatal P0 and P12 (2-3 animals each), were perfused with phosphate-buffered (PB) 4% paraformaldehyde. The brains were postfixed with the same fixative, cryoprotected with 30% sucrose, and sectioned at 60 μm. F-Spondin antisense riboprobe was labeled with digoxigenin-dUTP, or [35S]-dUTP (Boehringer-Mannheim) by in vitro transcription of a 2.4 kb fragment encoding mouse F-Spondin using T7 polymerase (Ambion).

Cold in situ hybridization was performed on free-floating sections essentially as described elsewhere (Alcántara et al., 1996; de Lecea et al., 1994). Control experiments included hybridization with a sense riboprobe and omission of primary antibodies.

In situ hybridization with [35S]-dUTP-labeled single-stranded mindin antisense RNA probes was performed as described previously (Wilkinson et al., 1987). Sense probes were used as controls.

**Transfection and purification of mindin-His protein**

HEK 293 cells were maintained in DMEM containing 10% fetal calf serum and transfected with pSec-min-HIS using a modified calcium phosphate method. For each 10 cm plate of subconfluent (30% confluent) cells, 1 ml of a solution containing 0.45 ml of 0.1x TE (1 mM Tris pH 8.0, 0.1 mM EDTA), pH 8.0, 0.5 ml of 2x HBS (2.80 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4·2H2O, 12 mM dextrose, 50 mM Hepes), pH 7.15, 4 μl phosphatase buffer and 1 μl of 0.5 M CaCl2 was added to the growth medium. After 14 hours, the plates were washed once with PBS and 10 ml of growth medium were added. After 18 hours the medium was changed to 5 ml/plate OPTI-MEM. Conditioned medium was collected after 4-8 days. 2 ml of agarose-Talon (Clontech) were added to 40 ml of conditioned medium, and the protein was purified according to the manufacturer’s protocol. The...
precoated with poly-D-lysine and laminin, at

described above and plated on 96-well plates,

(Cheng and Flanagan, 1994).

histochemistry was performed as described

Mindin-AP, AP-Fsp/TSR or AP the SEAP

cut with a chopper and after incubation

containing Mindin-AP, TSR-AP or AP, cells

were only measured if the entire length of the

tip of its longest neurite. Neurite lengths

were visualized by indirect

neurites were visualized by indirect

Developmental Studies Hybridoma Bank),

Triton X-100 and stained using mAb 3A10

which recognizes a neuronal filament-

associated protein and serves as a marker for

fine neurites. Neuronal cell bodies and

were visualized by indirect

immunofluorescence on a Zeiss Axioplan

microscope. Neurite lengths were measured

as the distance from the edge of the soma

(sharply defined by 3A10 fluorescence) to

the tip of its longest neurite. Neurite lengths

were only measured if the entire length of the

neurite could be unambiguously identified.

Binding experiments

Hippocampal cells were dissociated as described above and plated at a density of 200,000 cells/35 mm dish precoated with poly-D-lysine and laminin. The cells were cultured at 37°C in 5% CO₂ for 20 hours in the same medium as for the outgrowth assay. After incubation with conditioned medium containing Mindin-AP, AP, or AP, cells were processed for AP histochemistry as described (Cheng and Flanagan, 1994).

P5 rat brain sections (400 µm) were cut with a chopper and after incubation with conditioned medium containing Mindin-AP, AP-Fsp/TSR or AP the SEAP histochemistry was performed as described (Cheng and Flanagan, 1994).

For quantitative analysis, cells were dissociated as described above and plated on 96-well plates, precoated with poly-D-lysine and laminin, at a density of 3000 cells/well. The cells were cultured at 37°C in 5% CO₂ for 40 hours in the same medium as for the outgrowth assay. The cells were incubated with conditioned medium containing Mindin-AP, Mindin-T-AP, AP-Min/TSR or AP-Fsp/TSR1-4. Increasing concentrations of heparin or NaCl were added to the conditioned medium during the incubation period. The binding procedure was performed as described (Cheng and Flanagan, 1994). Bound, heat-stable alkaline phosphatase was detected by incubation with p-nitrophenyl phosphate for 20 hours at room temperature.

RESULTS

Isolation and characterization of rat mindin

The F-spondin family of secreted extracellular matrix-associated molecules include: F-spondin, expressed in the embryonic vertebrate floor plate and in early Schwann cells (Klar et al., 1992a, b; Ruiz-Il-Altaba et al., 1993; Higashijima et al., 1997;}

Fig. 1. Comparison of the rat Mindin, the zebrafish Mindin1 and Mindin2, the Drosophila M-spondin and the rat F-spondin proteins. (A) Schematic representation of the domain structure of F-spondin and Mindin proteins. Black box, the signal sequence; yellow box, the spondin domain; red boxes, the thrombospondin type I repeats (TSRs); turquoise box, the reelin domain. (B) Amino acid alignment of the rat Mindin, the zebrafish Mindin1 and Mindin2, the Drosophila M-spondin and the rat F-spondin proteins. Upper case letters are homologous amino acids. Dots are mismatches. Underlined are the amino acids that correspond to the degenerate PCR primers.
Burstyn-Cohen et al., 1998, 1999), mindin1 and mindin2, expressed in the zebrafish floor plate (Higashijima et al., 1997); and the Drosophila M-spondin, expressed mainly in muscle cells (Umemiya et al., 1997). All of these proteins contain thrombospondin type 1 repeat(s) (TSR), and novel domains designated FS1 and FS2 (the FS1 and FS2 will be referred to as the spondin domain throughout the paper) (Higashijima et al., 1997). The similarity between F-spondin and Mindin also extends to their expression domains. In the embryonic spinal cord of zebrafish, two F-spondin genes (F-spondin1 and F-spondin2) and two mindin genes (mindin1 and mindin2) are expressed on the floor plate (Higashijima et al., 1997). In order to clone the rodent mindin homologue, we performed PCR on rat genomic DNA using degenerate primers corresponding to two highly conserved 5-amino-acid sequences, PSPDW and rat genomic DNA using degenerate primers corresponding to two highly conserved 5-amino-acid sequences, PSPDW and DATGD, both of which are found in the spondin domain. Using PCR and subsequent 3' RACE and cDNA library screening, we identified several cDNA clones, one of which contained a 4 kb insert. Altogether 2390 bp from the 3' end were sequenced.

The mindin cDNA contains a single long open reading frame that starts with a methionine codon at nucleotide 512 associated with a conventional translation initiation sequence (Kozak, 1984) and ends with a TGA stop codon at nucleotide 1504 (Fig. 1). No in-frame methionine codons were found upstream of the putative translation initiation site. Sequences 5' of the initiation site contain stop codons in all three reading frames. Translation of the open reading frame of mindin predicts a protein of 330 amino acids with a calculated molecular mass of 36,174 Da (Fig. 1). Hydrophobicity analysis (Kyte and Doolittle, 1982) indicates that the protein has an N-terminal hydrophobic leader sequence with a consensus signal peptide cleavage site (von Heijne, 1985). No other long stretches of hydrophobic residues were observed, suggesting that the protein does not possess a transmembrane spanning domain. The predicted size and the domain organization of rat Mindin is identical to that of the zebrafish Mindins (Fig. 1), with a hydrophilic signal sequence in the amino terminus, a spondin domain (amino acids 34-239) and a TSR domain (amino acids 272-330). The homology between the rat Mindin protein and Mindin1 and Mindin2, over the entire length of the protein, is 60% and 47%, respectively. The spondin domain of rat Mindin shares 44% homology with the spondin domain of M-spondin whilst the TSR domain of M-spondin and rat Mindin are 34% homologous. F-spondin shares 37% (spondin domain) and 29% (TSR domain) homology with Mindin (Fig. 1). Thus the rat mindin is probably the homologue of the zebrafish mindin1 gene. The accession number is AF155196 (GenBank). Subsequent low stringency screening of a brain cDNA library and degenerate PCR with brain and embryonic spinal cord cDNA did not reveal any additional genes.

Expression pattern of mindin
To examine the expression of mindin in adult tissues, northern blotting with poly(A)+ RNA derived from rat tissues was performed. A prominent band of 2 kb was evident in liver, spleen, placenta and lung, indicating high levels of expression, whilst only low levels were observed in brain, heart, kidney, muscle and thymus (Fig. 2A). Fainter bands of 2.3 kb (liver, lung and muscle), 3 kb (lung), 5 and 5.5 kb (lung and spleen) were also detected. No signal was detected in the testes. A developmental northern blot of the hippocampus demonstrated that the high molecular weight mRNAs are downregulated during development (Fig. 2C). Comparison of the quantity of mindin and F-spondin mRNAs reveals that, except in the lung, they are inversely proportional. Where there are high levels of F-spondin (brain and kidney), there are low levels of mindin and conversely, where there are high levels of mindin (liver, spleen and placenta), there are low levels of F-spondin.

To determine the spatial and temporal patterns of mindin mRNA during embryogenesis, we performed in-situ hybridization analyses in rat and mouse embryos (Fig. 3). No mindin mRNA could be detected at embryonic day 12 (E12). Unlike the zebrafish mindins, expression of the rat gene was not detected in the floor plate at any time, but was predominantly expressed in forebrain structures, particularly in the cerebral cortex. Thus, at E15, weak levels were observed in the neocortex (data not shown). Starting at E18, mindin was also detected in the hippocampus, in both pyramidal neurons in the CA1-3 fields and in the granule cells of the dentate gyrus. Mindin mRNA was found in both the hippocampus and neocortex and levels were sustained after birth, with highest levels being detected at postnatal days 0 (P0) (Fig. 3A), 12 (P12) (Fig. 3B) and in the adult (data not shown). In the postnatal and adult neocortex, mindin was detected throughout the cortical layers (Fig. 3A,B). In both the developing and adult brain, mRNA levels were higher in the hippocampus than in the neocortex.

Expression pattern of F-spondin in the brain
Since the expression domains of F-spondin and mindin in
zebrafish overlap, we analyzed the rodent F-spondin pattern of expression and compared it to that of mindin. At E10-E11, F-spondin was expressed almost exclusively in the floor plate of the spinal cord and in the retina (data not shown). At later stages (E12-E18), high levels of expression were detected in the ventral midline of the hindbrain, midbrain and diencephalon (Fig. 3C), and in selected dorsal areas of these brain regions, including the cerebellum (data not shown). In the forebrain, increasing levels of F-spondin were found between E14 and E18 in the septum (Fig. 3D), olfactory bulb (Fig. 3E), striatum (Fig. 3C) and cerebral cortex (Fig. 3C,F). At E18, F-spondin was prominent in layer V of the neocortex and in the pyramidal neurons of the CA1-CA2 fields of the hippocampus (Fig. 3F). Weaker hybridization signals were detected in the dentate granule cells and in the remaining neocortical layers (Fig. 3F). The expression in CA1 and CA2 persists until adulthood. In early postnatal brains (P0-P12), F-spondin expression persisted in several layers of the neocortex (data not shown) as well as in the hippocampus and dentate gyrus. In the adult, the levels of expression were lower than at postnatal stages with transcripts being detected in several neocortical layers, in the dentate gyrus and in the hippocampus proper (Fig. 3G).

To study the profile of expression of mindin and F-spondin, a quantitative northern blotting analysis was performed (Fig. 2C,D). Equal amounts of total RNA, extracted from E17, E19, P0, P5 and adult rat hippocampus, were analyzed. Mindin expression was detected in the E17 hippocampus, but not in the E17 neocortex. The expression of mindin peaked at E17-E19 in the hippocampus. Levels of expression from P0 to adulthood appeared stable. Low levels of expression were detected in the E17 hippocampus and cortex. The level of F-spondin hippocampal expression increased until P0, and remained stable at P5 and adulthood.

The present expression analysis demonstrates overlapping patterns of expression of mindin and F-spondin in the rodent brain, although the pattern of mindin expression appears to be more restricted. In addition to the cerebral cortex, the expression of F-spondin is extended to other brain regions such as the striatum, the septum (Fig. 3), and the cerebellum (data not shown).

The structural resemblance and co-expression of mindin and F-spondin on the zebrafish floor plate, in the neocortex and in the hippocampus of the rodent brain, suggests that both proteins share similar biological roles, and thus one might be redundant. In other somatic tissues (not including the lungs) Mindin and F-spondin may complement each other, as indicated by their differentiated expression.

**Mindin and F-spondin receptor(s) are expressed on hippocampal neurons**

The onset of expression of Mindin and F-spondin in the hippocampus at prenatal stages coincides with the growth and maturation of the main hippocampal afferents and with the development of the mossy fibers (the axons that extend from the granule cells) (Supèr and Soriano, 1994; Supèr et al., 1998a,b). To test whether hippocampal neurons express a receptor(s) that binds Mindin and F-spondin, we fused the coding region of both Mindin (Mindin-AP), the spondin domain of mindin (Min(-T)-AP) and the TSR domain of F-spondin (AP-Fsp/TSR) to alkaline phosphatase (AP), a readily detectable histochemical label (Fig. 4A) (Flanagan and Leder, 1990), and expressed the resulting chimeric proteins in HEK 293 cells. The proteins could be detected by western blotting in conditioned medium from transfected cells as bands of 100 kDa, 95 kDa and 110 kDa, respectively (Fig. 4B-D). This is consistent with the combined sizes of Mindin, the spondin domain of mindin, and the TSR domain of F-spondin with AP.
When these media were applied to dissociated cultures of rat E17 hippocampal neurons, AP reactivity could be detected on the cell bodies, axons, dendrites and growth cones of the cultured neurons (data not shown). In contrast, control cultures incubated with secreted alkaline phosphatase (SEAP), also expressed in HEK 293 cells, showed no detectable binding (data not shown).

Because the above dissociated hippocampal cultures contain distinct classes of neurons (e.g. pyramidal and granule cells, Cajal-Retzius cells, etc), we incubated P5 rat brain slices with the AP fusion proteins in order to characterize which class of neurons exhibit binding activity. In addition to other brain regions, Min(-T)-AP, Mindin-AP and AP-Fsp/TSR specifically bound to the granule cell layer in the dentate gyrus and to the pyramidal neurons in the CA1-CA3 subfields (Fig. 5A-C). Control slices incubated with SEAP did not show any staining (Fig. 5D). These results indicate that pyramidal and granule cells express both mindin and F-spondin mRNAs as well as their putative receptor(s).

To examine the relative contribution of the different Mindin and F-spondin domains to the binding, we generated domain-specific AP fusion proteins: AP-Min/TSR (Fig. 4A,C) containing the TSR repeat of Mindin (Fig. 4A,C), and a fusion protein containing TSR repeats 1-4 of F-spondin fused to AP (AP-Fsp/TSR1-4 (Fig. 4A,D). All the fusion proteins bound to hippocampal neurons (Fig. 5H,I). In order to quantify the binding of the various mindin domains, we used soluble color substrate (p-nitrophenylphosphate). The amount of protein required for binding was in the µg range, suggesting that the binding is mediated by a low affinity receptor(s). The affinities of the different domains were ranked according to their AP activity. The affinity of the Mindin spondin domain (Min(-T)-AP) was similar to that of the intact Mindin protein (Mindin-AP) (Fig. 5H,I) whilst the affinity of the Mindin TSR domain (AP-Min/TSR) was considerably less than that of the intact Mindin (20%) (Fig. 5H,I). This implies that the relative contribution of the Mindin TSR domain for receptor binding is low. The receptor affinity of AP-Fsp/TSR1-4 protein was higher than that of the the Mindin TSR protein (Fig. 5H,I). This difference in affinity might be due to the additional TSR repeats (4 versus 1) in the F-spondin fusion protein. Accordingly, a fusion protein containing only the first TSR of F-spondin binds hippocampal neurons with a lower affinity than the four TSR fusion protein (data not shown). The binding of Mindin and F-spondin TSR domain proteins to hippocampal neurons was sensitive to salt (Fig. 5H) and heparin (Fig. 5I). Full inhibition of the binding was evident at 400 mM NaCl and 0.5 µg/ml heparin. This suggests that binding is mediated by electrostatic interactions, probably via interactions with glycosaminoglycans (Klar et al., 1992). The binding of the

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**Fig. 4.** Expression and purification of Mindin and F-spondin proteins. (A) Schematic representation of the expression vectors of F-spondin and Mindin. Black box, the signal sequence; yellow box, the spondin domain; red boxes, the thrombospondin type I repeat (TSRs); turquoise boxes, the reelin domain; gray box, the alkaline phosphatase gene; green box, the myc+6x Histidine cassette. (B) Western blot with R10 antibody, of conditioned medium of HEK 293 cells transfected with Mindin-HIS and Min(-T)-HIS and Min(-T)-AP plasmids. A 38 kDa, 30 kDa and 90 kDa protein was detected, respectively. (C) Western blot of F-spondin proteins. The TSR domain and the AP-Fsp/TSR fusion gene yielded a 40 and 110 kDa protein, respectively, recognized by the R2 Ab. A 95 kDa protein is detected by 9E10 mAb in the AP-Fsp/TSR1-4 transfected cells. A 100 kDa protein in the Mindin-AP transfection and an 80 kDa protein in the AP-Min/TSR transfection. (D) Western blot of F-spondin proteins. The TSR domain and the AP-Fsp/TSR fusion gene yielded a 40 and 110 kDa protein, respectively, recognized by the R2 Ab. A 95 kDa protein is detected by 9E10 mAb in the AP-Fsp/TSR1-4 transfected cells. (E) Purified Mindin and F-spondin proteins. Mindin-HIS protein, Mindin-AP, reeso-HIS and Fsp/TSR-HIS proteins were obtained from affinity-purified medium from transfected HEK 293 cells. A single band is observed in an SDS-PAGE gel stained with Coomassie Blue. The positions of marker proteins are shown.
Fig. 5. Binding of F-spondin and Mindin alkaline phosphate fusion proteins to hippocampal and sensory neurons. (A-D) Binding of AP fusion protein to slices of P5 rat brain. (E-G) Binding of AP fusion proteins to cultured rat E14 DRG neurons. The images to the left (E-G) are phase contrast, showing the cultured cells, and to the right (E'-G') are bright field, showing the stained cells. The control protein, secreted alkaline phosphatase (SEAP), did not bind to hippocampal (D) and to DRG neurons (E). F-spondin fusion protein (AP-Fsp/TSR) stained the pyramidal cell layer of the hippocampus and the granule cell layer of the dentate gyrus (A), and E14 DRG neurons (F). The Mindin-AP fusion protein stained the pyramidal cell layer of the hippocampus and the granule cell layer of the dentate gyrus (B), and E14 DRG neurons (G). Bar, 200 μm (A-C); 100 μm (D); 20 μm (E-G). (H-K) Blocking of the binding of mindin and F-spondin domain AP fusion proteins to hippocampal (H,I) and DRG (J,K) neurons. Neurons were cultured on a laminin substrate and grown for 14 hours (DRG) or 40 hours (hippocampal). Binding of the fusion protein was performed in the presence of increasing amounts of NaCl (H,J) or heparin (I,K).
F-spondin and Mindin promote the outgrowth of hippocampal neurons

To examine the biochemical and functional characteristics of the Mindin protein we generated an epitope-tagged derivative, Mindin-HIS, that contained the myc oncogene epitope and a 6-histidine (6×His) cassette fused to the carboxyl terminus of the protein (Fig. 4A). The cDNAs encoding Mindin-His were cloned into a mammalian expression vector and transfected into HEK 293 cells. The conditioned media of the transfected cells were analyzed by western blotting with the 9E10 mAb. A band corresponding to 35 kDa was evident (Fig. 4C). Thus, under these in vitro conditions, Mindin is synthesized in a secreted form.

To examine the in vivo localization of Mindin, we generated a rabbit antibody (R10) against the amino acids 50-166 fragment of the protein. The R10 specifically detected the 35 kDa protein that was detected by anti-myc antibodies, in the conditioned medium of transfected HEK 293 cells (Fig. 4B). However, the R10 serum failed to detect the Mindin protein in immunohistochemistry of brain sections (data not shown).

Having established that Mindin is a secreted protein, we then went on to determine whether Mindin, or F-spondin, promotes adhesion and neurite outgrowth of hippocampal neurons. To generate substrates of recombinant Mindin-His protein, conditioned media of transfected HEK 293 cells were affinity-purified (Fig. 4E) and immobilized onto nitrocellulose (Lemmon et al., 1989). F-spondin (Burstyn-Cohen et al., 1998), laminin and bovine serum albumin (BSA) substrates were prepared in a similar fashion. A suspension of dissociated rat E17 hippocampal neurons was plated onto these substrates, and neurite outgrowth was measured after 40 hours in culture.

As shown in Fig. 6, the number of cells that adhered to Mindin was significantly greater than the number of cells that adhered to BSA. Moreover, the number of cells that adhered to F-spondin was about three times greater than those that adhered to Mindin (Fig. 6). Thus, it can be concluded that F-spondin serves as a better substrate for adhesion of hippocampal neurons than Mindin.

To test whether the adhesion of hippocampal neurons to Mindin is mediated by either the spondin or TSR domain of the Mindin protein, we expressed two truncated forms of the Mindin protein, Min(-T)-His, lacking the TSR domain, but containing the spondin domain, and the AP-Min/TSR, lacking the spondin domain, but containing the TSR repeats. These were fused in-frame to the myc epitope and 6×His. The Min(-T)-His protein was detected in the conditioned medium of HEK 293 cells as a 30 kDa protein by both the 9E10 mAb (Fig. 4C) and the R10 serum (Fig. 4B). When purified Min(-T)-His protein and the AP-Min/TSR protein (Fig. 4F) were used as substrates, the number of adhered hippocampal cells was not significantly different from the number of cells that adhered to BSA (Fig. 6). We concluded that the intact Mindin is required to promote adhesion of hippocampal neurons.

To analyze the outgrowth-promoting activity of Mindin and F-spondin, cultured hippocampal neurons were stained after 2 days in vitro with the Tuj1 mAb, which recognizes class III β-tubulin, and stains both axons and dendrites of pyramidal and granular cells. As shown in Fig. 7B, F-spondin-His dramatically increased neurite outgrowth compared with BSA. Laminin, a well-known neurite outgrowth promoter (Baron Van Evercooren et al., 1982), exerted a neurite growth effect comparable to that of F-spondin (data not shown). The mean length of neurites grown on Mindin-His was about twice as short as that of F-spondin (Fig. 7B). In contrast, the truncated Mindin proteins had no effect on neurite outgrowth (data not shown). Taken together, this data implies that both F-spondin and Mindin promote adhesion and neurite outgrowth of hippocampal neurons, with F-spondin being the more potent. It is conceivable that both the spondin domain and the TSR domain of Mindin are required in combination to elicit adhesion and outgrowth. However, when using the purified TSR domain of F-spondin in an outgrowth assay, the extent of outgrowth was similar to that elicited by the intact protein (data not shown). This implies that the TSR domain of F-spondin is sufficient for promoting outgrowth.

In order to determine whether F-spondin and Mindin promote dendritic or axonal growth differentially, cultured hippocampal neurons incubated with F-spondin or Mindin were immunostained with the Tuj1 (TRITC) mAb together with an anti-MAP2 antibody (FITC) (a well-known dendritic marker). The data shows that both F-spondin and Mindin promote the growth of single-labeled Tuj1-positive axonal processes and several short dendrites (Tuj1/MAP2-positive
These experiments demonstrate that, in dissociated cell cultures, F-spondin and Mindin promote both axonal and dendrite growth. When F-spondin and Mindin were immobilized on a permissive substrate (poly-L-Ornithine), dissociated hippocampal neurons extend longer axons than on control substrates. However, the extent of dendrite outgrowth was only moderate.

**Mindin promotes outgrowth of sensory neurons**

To test whether other known neuronal populations sensitive to F-spondin (Bursten-Cohen et al., 1998; Klar et al., 1992a) bind to the F-spondin and Mindin proteins, cultured E14 DRG neurons were incubated with the chimeric proteins. This resulted in the labeling of the cell bodies, axons and growth cones, in a manner similar to the staining of hippocampal neurons. DRG neurons appear to bind AP-Fsp/TSR (Fig. 5F) with the same affinity as Mindin-AP (Fig. 5G). SEAP did not bind to the cells at all (Fig. 5E). Sensitivity to NaCl and heparin was also tested. AP-Min/TSR and AP-Fsp/TSR1-4 (the TSR domain of F-spondin) binding was abolished in the presence of NaCl and heparin.

**Fig. 7.** Outgrowth-promoting activity of Mindin and F-spondin proteins. (A) Hippocampal neurons were grown on F-spondin stained with Tuj-1 mAb (TRITC) and MAP2 (FITC). One long process is labeled with Tuj-1, and several short processes are double labeled. Bar, 20 μm. (B) Outgrowth of E17 hippocampal neurons on a substrate of F-spondin and Mindin proteins. The neurons were stained with Tuj-1 mAb. For each Tuj-1-positive neuron, the neurite length was measured or, if no neurite was seen, considered to be 0. The graph shows the percentage of neurons with neurites longer than a given length (in μm). (C, D) Outgrowth of E14 DRG neurons on a substrate of purified Mindin (C) and Min(-T)-His proteins (D). Outgrowth on purified reelin/spondin domain protein- reespo-His is blocked by the spondin domain-specific antibody R5 (E). Outgrowth on purified TSR domain protein (Fsp-TSR-His) is blocked by the TSR domain-specific antibody R2 (F). The neurons were stained with the 3A10 mAb.
of both salt and heparin (Fig. 5J,K). This implies that the interaction between hippocampal and DRG neurons with the TSR domains is similar. In contrast, the binding characteristics of the Mindin spondin domain to DRG neurons was different from its binding characteristics to hippocampal neurons. In the presence of NaCl, the binding was totally abolished (Fig. 5J), whereas heparin reduced the binding by only 50% (Fig. 5K). This indicates that, unlike hippocampal neurons, DRG neurons are recognized by the spondin domain via electrostatic interactions. These are probably not mediated by glycosaminoglycan (since they are not fully blocked by heparin).

The binding assays with the Mindin-AP protein clearly demonstrate that Mindin binds to embryonic sensory neurons. To test whether Mindin can promote outgrowth of sensory neurons, we cultured suspensions of E14 DRG cells on Mindin-His and Min(-T)-His substrates. As shown in Fig. 7C, the outgrowth promoted by Mindin-His was substantially greater than that promoted by BSA and was comparable to that observed with Laminin(s) and F-spondin-His (data not shown). Furthermore, the response was dose-dependent, with 20 μg/ml of Mindin-His being more potent than 10 and 5 μg/ml, respectively (Fig. 7C). 1 μg/ml of Mindin-His had no apparent effect. Min(-T)-His also promoted outgrowth of DRG neurons, but to a lesser extent (70%) than Mindin-His (Fig. 7D). These experiments show that the Mindin spondin domain promotes outgrowth of embryonic sensory neurons, but not hippocampal neurons.

To test whether the F-spondin isolated domains promote DRG neurite outgrowth, we generated F-spondin fragments containing an epitope tag. Specifically, Reespo-His and TSR-His contain a myc tag and a cassette of six histidines (Burstyn-Cohen et al., 1999) (Fig. 4A,E). Both fragments promote outgrowth of DRG neurons (Fig. 7E,F). The purified domains were responsible for the outgrowth since it was specifically blocked with domain-specific antibodies: R2 inhibited the outgrowth caused by the TSR domain protein (Fig. 7E), and R5 inhibited the outgrowth caused by the reespo domain protein (Fig. 7F). Conversely, the R2 Ab did not inhibit the outgrowth caused by the reespo substrate. Likewise, R5 did not inhibit the outgrowth caused by the TSR substrate (data not shown).

These results demonstrate that the spondin domain of F-spondin and Mindin can promote the outgrowth of DRG neurons, and they corroborate the observation that an F-spondin domain-specific antibody blocks the outgrowth of sensory neurons on a substrate of F-spondin (Burstyn-Cohen et al., 1998).

**DISCUSSION**

In the current study we have cloned the rat homologue of the mindin gene. Mindin and F-spondin belong to a family of secreted adhesion proteins that share similar structural properties. We show here that both genes share overlapping patterns of expression in the developing and adult brain, which suggests that they may also share similar functions. The present in vitro studies indicate that both F-spondin and Mindin promote adhesion and outgrowth of embryonic hippocampal and sensory neurons. We also demonstrate that both proteins bind to a putative receptor(s) expressed on both hippocampal and sensory neurons.

**The spondin and TSR domains promote differentially neuronal outgrowth in the DRG and the hippocampus**

F-spondin and Mindin are members of a small family of evolutionarily conserved, secreted adhesion molecules. In invertebrates a single F-spondin gene was identified in C. elegans (Wilkinson et al., 1987) and two in Drosophila (A. Nose, unpublished). Only a single mindin-like gene, M-spondin, was identified in the fly (Umemiya et al., 1997). The structural similarities between the vertebrate and the invertebrate F-spondin is clear. All of the F-spondin genes contain a reelin domain at the amino terminus, followed by a spondin domain, and multiple TSR repeats (five in invertebrate and six in vertebrate) at the carboxyl terminus (Wilkinson et al., 1987; A. Nose, unpublished). Similarly the structure of the mindin genes, composed of a spondin domain and one TSR domain, is conserved. In rodent, only one mindin and one F-spondin gene was identified. The search for other F-spondin/mindin-like genes by either degenerate PCR or low stringency screening did not reveal any other genes. Likewise, screening for human homologous genes by either genomic library screening (our unpublished results) or databank searching (mindin data not shown) revealed only one mindin and one F-spondin gene. The two mindin and F-spondin genes present in zebrafish are most likely to be the result of an additional chromosomal duplication event, not seen in mammals (Wittbrodt et al., 1998; Postlethwait et al., 1998). Thus, it is probable that Mindin and F-spondin are the only representatives of this protein family in mammals.

We had previously demonstrated that the F-spondin protein promotes outgrowth of sensory and commissural neurons (Klar et al., 1992; Burstyn-Cohen et al., 1998, 1999). In the current study we have shown that Mindin can also promote outgrowth of sensory neurons and that both Mindin and F-spondin are potent neurite outgrowth-promoting proteins for dissociated hippocampal neurons in vitro. To examine the relative contribution of each domain of the Mindin protein we generated truncated Mindin proteins. The outgrowth assay demonstrated that both the TSR and the spondin domains of Mindin are required for the promotion of outgrowth of hippocampal neurons. Binding assays with Mindin-domain AP fusion proteins demonstrated that the affinity of the spondin domain is higher than that of the TSR domain. However, the spondin domain does not promote adhesion. It is conceivable that the Mindin spondin domain mediates the binding to hippocampal neurons, which in turn might enhance the binding of the TSR domain to the neurons. This, would then elicit adhesion and outgrowth. This hypothesis is supported by the observation that multiple F-spondin TSR domains alone can promote outgrowth, even in the absence of the reelin/spondin domain. Outgrowth experiments with thrombospondin have shown that it too can promote outgrowth of hippocampal neurons (Osterhout et al., 1992). Moreover, an antibody that recognizes the TSR domain can block the adhesion and outgrowth activity of thrombospondin (Osterhout et al., 1992). Alternatively, the binding of Mindin might be mediated by the TSR domain, which recognizes abundantly expressed proteoglycan(s) on the axons, and the spondin domain would...
then mediate the outgrowth. This assumption is not supported by the observation that, in vitro, the spondin domain of Mindin does not support either adhesion or outgrowth. Hence, it is conceivable that the spondin domain interacts with other factors in vivo, eliciting other biological effects. Perturbation experiments, either in vivo or in organotypic culture, with domain-specific antibodies, should help to clarify the biological role of the F-spondin and Mindin domains. The different biochemical properties of the TSR domain binding and the spondin domain binding indicates that recognition of the spondin domain may be mediated by a specific receptor, distinct from that of the TSR domain receptor.

In contrast, the purified spondin domain of Mindin can promote outgrowth of sensory neurons. We have previously demonstrated, by using domain-specific antibodies, that the spondin domain of F-spondin is essential for promoting outgrowth of sensory neurons (Burstyn-Cohen et al., 1998). As opposed to sensory neurons, hippocampal neurons do not adhere or extend neurites on the Mindin spondin domain. The binding of the spondin domain to DRG neurons is biochemically different from the binding to hippocampal neurons. It is conceivable that the difference is attributed to different receptors. Spondin may interact hydrophobically with the receptor expressed on hippocampal neurons, whilst an interaction between it and the DRG might be electrostatic. The ‘hippocampal’ receptor mediates the activity of Mindin via its TSR domain, and the binding to the ‘DRG’ receptor mediates neurite outgrowth.

**Possible role of F-spondin and Mindin in hippocampal development**

The present study has shown that F-spondin and Mindin are highly expressed in the developing cerebral cortex, particularly in the hippocampus. Furthermore, our in vitro data indicate that both proteins exert dramatic effects on neurite outgrowth of hippocampal neurons, and that this region contains specific F-spondin and Mindin receptors and/or binding proteins. Adhesion molecules and extracellular matrix proteins have been found to be implicated in the development of the cerebral cortex, including the hippocampus, by regulating both neuronal migration and the growth of certain afferent systems. ECM molecules like laminin and J1/tenascin are also expressed in the hippocampus, and can promote neurite outgrowth of hippocampal neurons (Faisstner, 1997).

Recent studies have also shown that the reelin gene, encoding a large extracellular matrix protein, is essential for cortical development (D’Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995). Reelin is expressed very early in a subset of pioneer neurons, the Cajal-Retzius (CR) cells, which are present in the developing layer I of the neocortex and the outer marginal zone of the hippocampus (Alcántara et al., 1998; D’Arcangelo et al., 1995; Ogawa et al., 1995). The lack of this extracellular matrix protein dramatically alters cortical neuronal migration, resulting in abnormal lamination of the neocortex and hippocampus. In addition, as demonstrated both in vivo in the reeler mutant mouse and in vitro by antibody perturbation experiments (Borrell et al., 1998; Del Rio et al., 1997), Reelin is involved in regulating the growth and synaptogenesis of entorhinal afferents to the hippocampus. Our expression studies show that Reelin, F-spondin and mindin expression patterns do not overlap in the hippocampus and neocortex: whilst Reelin is expressed in superficial pioneer neurons, F-spondin and mindin transcripts are observed in the pyramidal and granule cells. Both mindin and F-spondin are expressed mainly from E17 onwards in the rodent cerebral cortex, suggesting that it is unlikely that these genes are involved in neuronal migration, at least for the earliest neuronal cohorts that are generated several days before (Supér et al., 1998a). However, the coexpression of both proteins and of their putative receptors within the main cellular layers of the hippocampus, raises the possibility that F-spondin and Mindin may act as local stopping signals for migrating neurons, thereby participating in the arrangement of the main cellular layers of the hippocampus, i.e. the pyramidal and granule cell layers.

The present in vitro studies indicate that F-spondin and Mindin may regulate both dendritic and afferent axonal growth in the developing hippocampus. The developmental expression of both genes in the hippocampus, peaking at perinatal stages coincident with the ingrowth, targeting and maturation of several hippocampal afferent (Supér et al., 1998a,b; Supér and Soriano, 1994), supports this notion. In addition, Mindin, which is expressed postnatally at high levels in the CA3, might also be involved in the growth of the mossy fiber projection to this region. Future studies, involving both protein perturbation experiments and deficient mouse models, are necessary to elucidate the exact functions of F-spondin and Mindin in the regulation of axonal growth in the hippocampus.

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