

AMIGO, a transmembrane protein implicated in axon tract development, defines a novel protein family with leucine-rich repeats

Juha Kuja-Panula,¹ Marjaana Kiiltomäki,¹ Takashi Yamashiro,² Ari Rouhiainen,^{1,3} and Heikki Rauvala¹

¹Department of Biosciences, Institute of Biotechnology, Neuroscience Center, and ²Developmental Biology Program, Institute of Biotechnology, University of Helsinki, Helsinki 00014, Finland

³Finnish Red Cross Blood Transfusion Service, Helsinki 00310, Finland

Ordered differential display identified a novel sequence induced in neurons by the neurite-promoting protein amphoterin. We named this gene amphoterin-induced gene and ORF (AMIGO), and also cloned two other novel genes homologous to AMIGO (AMIGO2 and AMIGO3). Together, these three AMIGOs form a novel family of genes coding for type I transmembrane proteins which contain a signal sequence for secretion and a transmembrane domain. The deduced extracellular parts of the AMIGOs contain six leucine-rich repeats (LRRs) flanked by cysteine-rich LRR NH₂- and COOH-terminal domains and

by one immunoglobulin domain close to the transmembrane region. A substrate-bound form of the recombinant AMIGO ectodomain promoted prominent neurite extension in hippocampal neurons, and in solution, the same AMIGO ectodomain inhibited fasciculation of neurites. A homophilic and heterophilic binding mechanism is shown between the members of the AMIGO family. Our results suggest that the members of the AMIGO protein family are novel cell adhesion molecules among which AMIGO is specifically expressed on fiber tracts of neuronal tissues and participates in their formation.

Introduction

Development of the nervous system with billions of connections is one of the most complex and fascinating phenomena in nature. One key feature in this event is the guidance of the neuronal growth cones to their appropriate targets. A wide variety of soluble, matrix, and cell surface molecules have been found to be involved in axonal growth and in association of axons to form mature fiber tracts (for review see Tessier-Lavigne and Goodman, 1996; Drescher et al., 1997).

Currently, Ig superfamily proteins form the most diverse and studied class of molecules, and have been shown to participate in contact-dependent regulation of neurite out-

growth, axon guidance, and synaptic plasticity (for review see Schachner, 1997; Walsh and Doherty, 1997; Stoeckli and Landmesser, 1998; Van Vactor, 1998). Extracellular proteins containing leucine-rich repeats (LRRs)* have also been shown to participate in axon guidance. For instance, Slit proteins containing LRR domains act as midline repellents for commissural axons through the Robo (Roundabout) receptor (Battye et al., 1999; Brose et al., 1999), and recently, Battye et al. (2001) showed that the interaction of Slits with their Robo receptors was due to LRRs found in Slits. Furthermore, Pusch et al. (2000) showed that the disease called X-linked congenital stationary night blindness (XLCSNB) maps to a gene that codes only the LRR-containing protein nyctalopin in retina. Recently, the receptor for axonal regeneration inhibitor Nogo (Chen et al., 2000) was found to be a GPI-linked cell surface protein in which the only recognizable motifs are the LRR domains (Fournier et al., 2001).

Amphoterin (also known as HMGB1) is a heparin-binding protein that was isolated from perinatal rat brain as a neurite outgrowth-promoting factor (Rauvala and Pihlaskari, 1987) enriched in the growth cones of neuronal cells. Amphoterin has been proposed to be an autocrine factor in invasive cell or growth cone migration due to binding to the cell surface

Address correspondence to Juha Kuja-Panula, Neuroscience Center, Viikinkaari 5, PO Box 56, University of Helsinki, Helsinki 00014, Finland. Tel.: 358-9-19159061. Fax: 358-9-19159068. E-mail: Juha.Kuja-Panula@Helsinki.fi; or Heikki Rauvala, Neuroscience Center, Viikinkaari 5, PO Box 56, University of Helsinki, Helsinki 00014, Finland. Tel.: 358-9-19159064. Fax: 358-9-19159068. E-mail: Heikki.Rauvala@Helsinki.fi

*Abbreviations used in this paper: AMIGO, amphoterin-induced gene and ORF; ChrB, chromogranin B; LRR, leucine-rich repeat; ODD, ordered differential display; RAGE, receptor for advanced glycation end products.

Key words: fasciculation; cell adhesion; neurite outgrowth; Ig superfamily; leucine-rich repeat

receptors (receptor for advanced glycation end products [RAGE] and sulphated glycan epitopes) and to activation of proteolysis of ECM through binding of plasminogen and its activators to amphoterin (for review see Rauvala et al., 2000; Muller et al., 2001).

To examine the role of amphoterin in cell motility, especially in neurite outgrowth, we searched for genes that are induced on amphoterin matrix by using mRNA differential display. A transcript that was prominently up-regulated on amphoterin-coated matrix was found to encode a novel protein. In this paper, we describe the cloning and functional characterization of this novel protein, named amphoterin-induced gene and ORF (AMIGO). Cloning of AMIGO gave us sequence data to clone two other related proteins (AMIGO2 and AMIGO3); together, these three proteins form a novel family of transmembrane proteins. The predicted amino acid sequences of the AMIGOs suggest that they are type I transmembrane proteins containing a signal sequence for secretion and a transmembrane domain. Interestingly, the extracellular part of the AMIGOs contains six LRRs flanked by cysteine-rich LRR NH₂- and COOH-terminal domains and by one Ig domain close to the transmembrane region. This twin motif structure defines the AMIGOs as members of both the Ig and the LRR superfamilies.

In this paper, we have characterized in more detail AMIGO, the member of the protein family that is highly expressed in the nervous system. We suggest that AMIGO mediates cell-cell interactions via a homophilic mechanism in the fiber tracts of the nervous system. In addition, we show that all members of the AMIGO family display homophilic binding, and that they also bind each other in a heterophilic fashion, suggesting a cooperative role during development and in adult animals.

Results

Identification of an amphoterin-induced transcript in hippocampal neurons

Ordered differential display (ODD; Matz et al., 1997) was used to search for amphoterin-induced genes in neurons. Comparison of ODD from embryonic d 18 rat hippocampal neurons grown on amphoterin- and laminin-coated plates revealed a transcript that was expressed more on amphoterin (Fig. 1 A). This expression difference was also confirmed with RT-PCR (Fig. 1 B).

To see whether the induction is mediated through the amphoterin receptor RAGE (for review see Rauvala et al., 2000; Schmidt et al., 2000), we also ligated RAGE by using affinity-purified anti-RAGE antibodies. The anti-RAGE matrix promoted prominent neurite outgrowth in hippocampal neurons, whereas a matrix coated with control rabbit IgG was inactive. The anti-RAGE-induced neurite outgrowth was specifically blocked by the peptide used for immunization (5 µg/ml), whereas the control peptide from a different part of RAGE did not have any effect on anti-RAGE-induced neurite outgrowth (unpublished data).

Expression analysis of the transcript was performed using hippocampal neurons on amphoterin-, anti-RAGE-, and laminin-coated plates. We compared the expression to chromogranin B (ChrB), for which the expression has been shown to be induced through RAGE signaling (Huttunen et

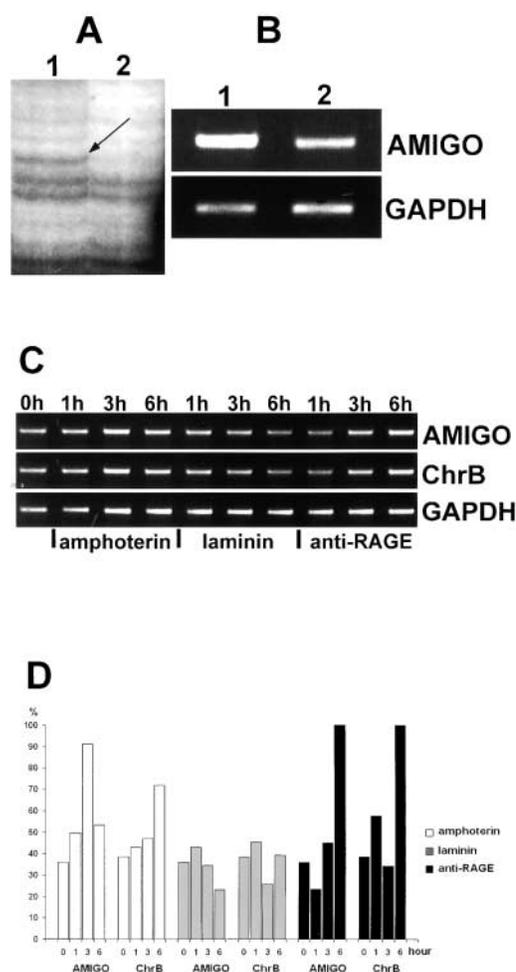


Figure 1. Identification of an amphoterin-induced gene in hippocampal neurons. (A) Analysis of ODD on gel electrophoresis, from which the band corresponding to AMIGO (see Fig. 2) was cut for sequencing (marked with arrow). Lane 1, sample from amphoterin matrix; lane 2, sample from laminin matrix. (B) AMIGO induction was confirmed using RT-PCR. Lane 1 contains an RT-PCR reaction from hippocampal neurons on amphoterin, and lane 2 on laminin. GAPDH was analyzed as a control. (C and D) AMIGO induction was confirmed to be RAGE dependent by culturing hippocampal neurons on amphoterin, laminin, and anti-RAGE antibody matrix. (C) RT-PCR experiment at different time points for AMIGO, chromogranin B (ChrB), and GAPDH. (D) GAPDH-normalized gene expression values for AMIGO and ChrB from picture C.

al., 2002). Interestingly, the expression patterns were quite similar for these two genes; both were induced on amphoterin and anti-RAGE matrix, whereas on laminin there was no induction (Fig. 1, C and D). Expression of the transcript identified by ODD is thus induced by RAGE (but apparently not by integrin) signaling.

Cloning of a novel family of transmembrane proteins containing a tandem array of LRRs and an Ig domain (AMIGO, AMIGO2, and AMIGO3)

The sequence of the partial transcript did not give homology to any previously cloned genes. By using the 5' RACE method (Matz et al., 1999), the cDNA encoding the whole coding sequence was cloned (Fig. 2 A). We named this differentially expressed gene AMIGO. Hydrophobicity profile

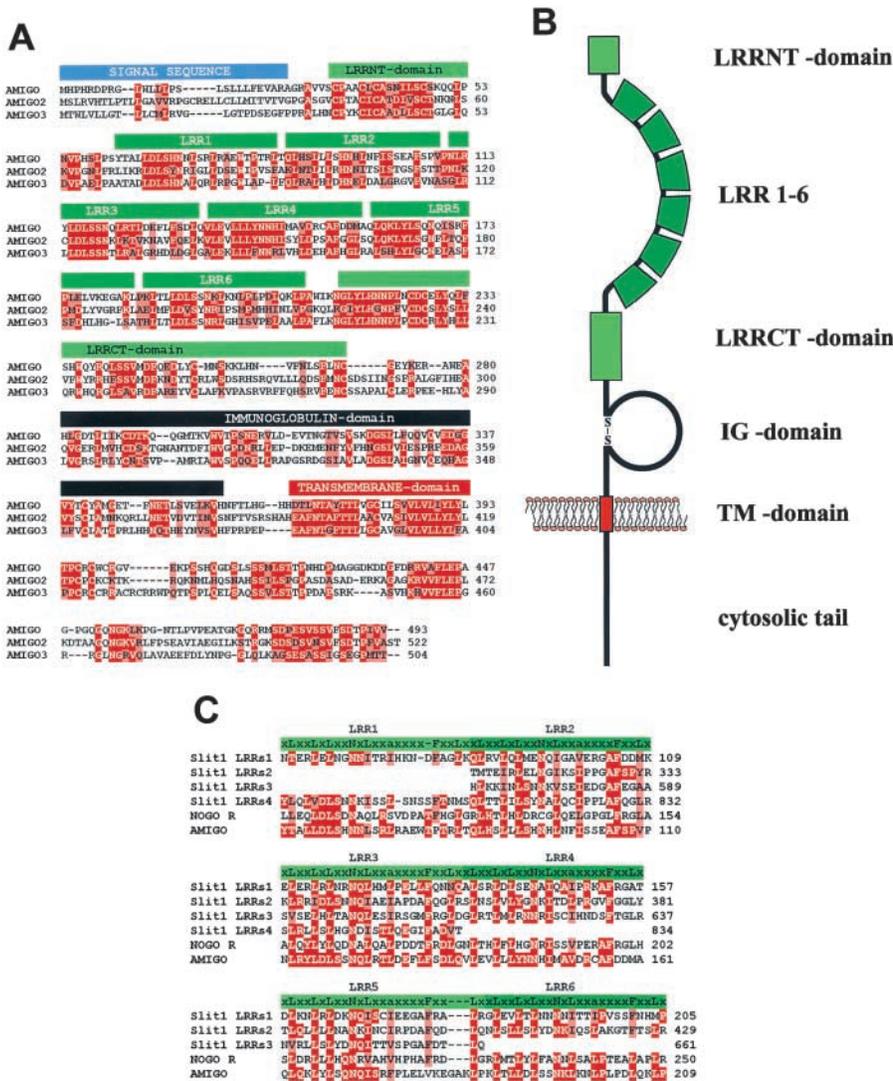


Figure 2. Primary structure of human AMIGO, AMIGO2, and AMIGO3.

(A) Alignment of the three AMIGOs where the identical amino acids between all AMIGOs are highlighted in red with white letters, and similar amino acids are highlighted in red with black letters. Different domains found in the AMIGOs are marked with colored boxes above the sequences. (B) Schematic view of the three AMIGOs. (C) Multiple alignment for the LRR areas of Slit1, Nogo receptor, and AMIGO. The identical amino acids between Slit1 and Nogo receptor compared with the AMIGO are highlighted in red with white letters, and similar amino acids are highlighted in red with black letters. The consensus sequence of the six LRR motifs of the AMIGO are shown above the sequences.

analysis (Nielsen et al., 1997; SignalP V2.0.b2 software) revealed that the protein sequence of AMIGO contains a putative signal sequence and a putative transmembrane region. The deduced extracellular part of the protein contains six LRRs and one Ig domain. The deduced cytosolic part of the protein does not contain any known domains.

The human and mouse homologues of AMIGO were also cloned with the 5' RACE method by using data from the rat AMIGO sequence and from EST sequences. The sequences of the rat, mouse, and human AMIGO are available from GenBank/EMBL/DDBJ under accession nos. AY237729, AY237008, and AY237007, respectively. Identity at the amino acid level between the rat and mouse AMIGO is 95%, and the murine sequences are 89% identical to the human AMIGO. In the extracellular part, the most conserved motifs between the murine and human AMIGO are the NH₂-terminal cysteine-rich domain and the LRRs 1–3. Interestingly, the whole transmembrane domain and the cytoplasmic tail are 100% identical between the murine and human AMIGOs.

By using homology search, we detected ESTs that gave homology but were not identical when compared to AMIGO. By using these EST sequences, we cloned two other novel genes that we named for convenience as

AMIGO2 and AMIGO3. The sequences of the mouse and human AMIGO2 are available from GenBank/EMBL/DDBJ under accession nos. AY237006 and AY237005, respectively. The sequences of the mouse and human AMIGO3 are available from GenBank/EMBL/DDBJ under accession nos. AY237004 and AY237003, respectively. The deduced amino acid sequences show that AMIGO2 and AMIGO3 have the same domain organization as AMIGO; they also contain a putative signal sequence for secretion and six LRRs flanked on both the NH₂- and COOH-terminal sides by cysteine-rich LRRNT and LRRCT domains. Like AMIGO, the deduced extracellular parts of AMIGO2 and AMIGO3 contain an Ig domain close to the transmembrane domain (for schematic picture of AMIGO, AMIGO2, and AMIGO3, see Fig. 2 B).

Similarity at the amino acid level between AMIGO to AMIGO2 is 48%, AMIGO to AMIGO3 is 50%, and AMIGO2 to AMIGO3 is 48%. The alignment for AMIGO, AMIGO2, and AMIGO3 shows that the most conserved regions between the three proteins are the LRRs, the transmembrane region, and some parts of the cytosolic tail (Fig. 2 A). The LRRs found in the AMIGOs can be described as a motif LX₂LXLX₂NX(L/I)X₂aX₄(F/L/I), in which “a” denotes

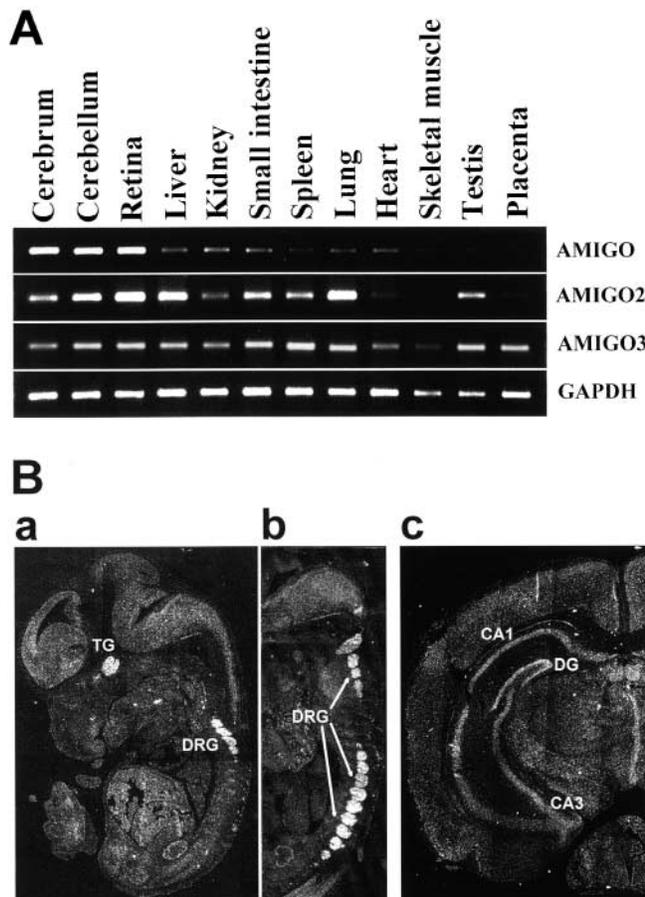


Figure 3. Expression of AMIGO mRNAs in adult and developing tissues. (A) RT-PCR mRNA analysis of AMIGO, AMIGO2, and AMIGO3 in different adult mouse tissues. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (B) In situ hybridization analysis of AMIGO mRNA. In E13 mouse embryo, the AMIGO mRNA expression is highest in the dorsal root ganglia (a and b; DRG) and in the trigeminal ganglion (a; TG). In the adult mouse cerebrum, the AMIGO expression is highest in the hippocampal formation where the most intense signal is seen in the dentate gyrus (c; DG). The pyramidal cell layers CA1–CA3 also express AMIGO (c).

an aliphatic residue and “X” denotes any amino acid; this motif resembles a typical LRR sequence often found in extracellular parts of animal proteins (Kajava, 1998).

We made BLAST searches by using the extracellular part of the AMIGO to see whether there were some homologous neuronal proteins. The best fits were found from Slit family of extracellular axon-guiding proteins (Whitford et al., 2002), and from the Nogo-66 receptor (Fournier et al., 2001; Fig. 2 C).

Expression of the gene family members in adult tissues

RT-PCR analysis of adult mouse tissues (Fig. 3 A) revealed that AMIGO is mainly expressed in the nervous tissues (cerebellum, cerebrum, and retina), although weak expression could be also detected in liver, kidney, small intestine, spleen, lung, and heart. AMIGO2 expression is most prominent in cerebellum, retina, liver, and lung. A lower AMIGO2 mRNA expression is also seen in cerebrum, kidney, small intestine, spleen, and testis. AMIGO3 mRNA expression could be detected in every tissue studied, showing no specific expression pattern compared with AMIGO or

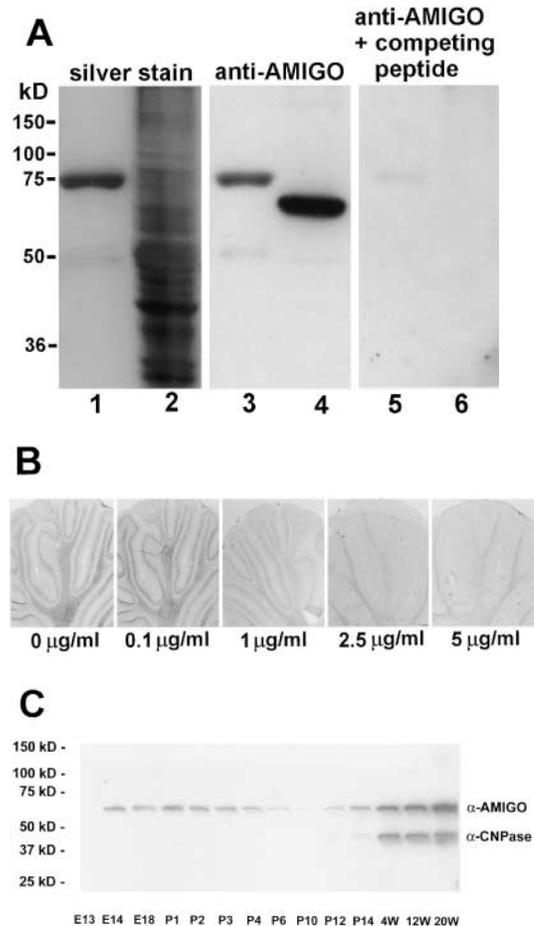


Figure 4. Characterization of the recombinant AMIGO-Ig fusion protein and of anti-AMIGO antibodies, and developmental expression of the AMIGO protein in brain. (A) The recombinant AMIGO-Ig fusion protein (lanes 1, 3, and 5) and protein lysates from adult brain (lanes 2, 4, and 6) were silver stained (lanes 1 and 2) or immunoblotted with rabbit anti-AMIGO antibodies (lanes 3–6). Anti-AMIGO identifies both the AMIGO-Ig fusion protein (lane 3) and one 65-kD band in rat brain lysate (lane 4). Binding of the antibodies to the band corresponding to AMIGO was inhibited by the peptide used for immunization (A; lanes 5 and 6). (B) Binding of the antibodies to tissue sections was also inhibited in a dose-dependent manner by the peptide (shown for the immunohistochemistry of the adult cerebellum). (C) AMIGO expression displays a dual character during brain development. Western blotting of AMIGO using crude brain extracts from different developmental stages reveals clear AMIGO expression during the late embryonic and perinatal development, starting at the E14 stage. The AMIGO expression is down-regulated during postnatal (P) stages P6–P10. The expression is again up-regulated between the stages P10 and P12, and remains high in the adult brain. The up-regulation coincides with the onset of myelination, as demonstrated by the comparison with the CNPase expression. The expression of AMIGO and CNPase displays a parallel increase during postnatal development. E, embryonic day; W, postnatal week.

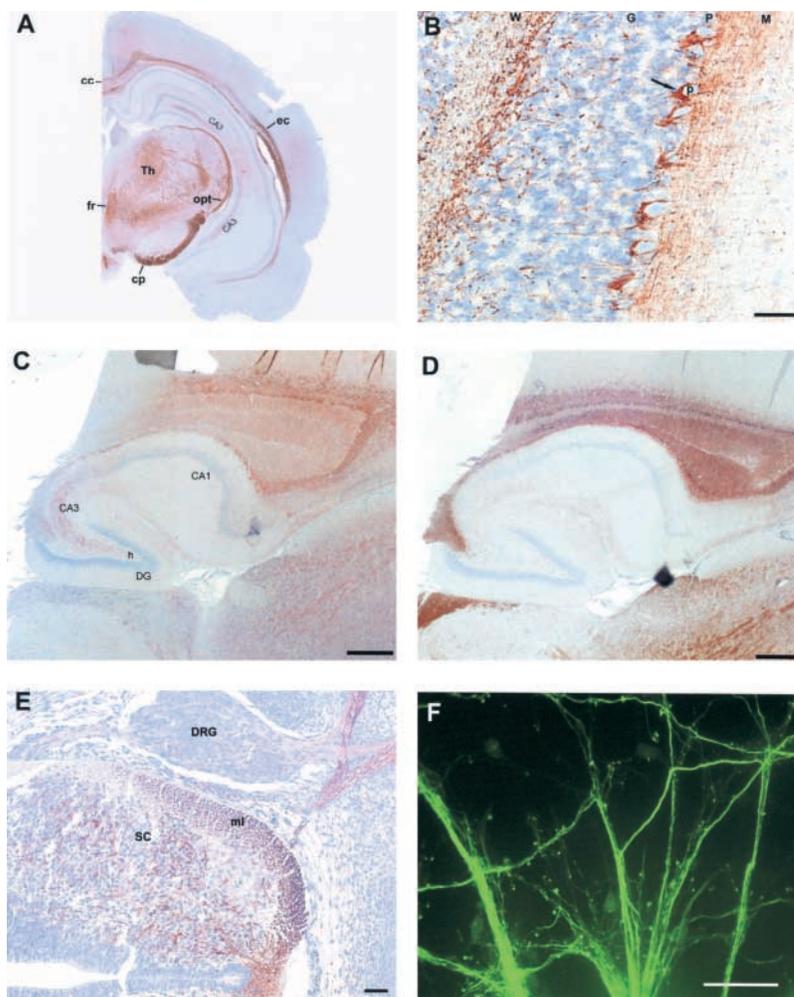
AMIGO2. Thus, it appears that AMIGO is essentially a nervous system-specific member of the protein family, and we focused on AMIGO in more detail in the present work.

Expression of AMIGO during development

The AMIGO mRNA expression was studied in more detail using in situ hybridization. The AMIGO antisense probe

Figure 5. AMIGO is localized to axonal fiber tracts in tissue and in cultured hippocampal neurons.

Immunohistochemical staining of rat tissues revealed that AMIGO is specifically expressed in the nervous system. In adult animals (A–D), AMIGO was particularly detected in nerve fibers and tracts. Myelinated fiber pathways were clearly stained for AMIGO in coronal sections of cerebrum (A). The presence of AMIGO in myelinated fibers is also demonstrated by the similar staining of AMIGO (C) and CNPase (D) around the hippocampus in sagittal sections. Nonmyelinated fibers were stained in the CA3 region of hippocampus (A and C). In the cerebellum (B), a distinct staining was located in nonmyelinated fibers on both sides of the Purkinje cell layer (P), as in the characteristic basket-like structure (arrow) formed by the basket cell axons around the Purkinje cell soma (p). Fibers in the cerebellar white matter (W) were also stained. In E15 embryo (E), immunostaining was observed in developing fiber tracts and nerves, like in the ventral part of the marginal layer (ml) of the spinal cord (SC) and in the nerves connecting to the dorsal root ganglion (DRG) and to the spinal cord. In cultured hippocampal neurons (F), AMIGO was also detected in neuronal processes by immunocytochemistry. CA1 and CA3, fields of the pyramidal cell layer in hippocampus; cc, corpus callosum; cp, cerebral peduncle; DG, dentate gyrus; ec, external capsule; fr, fasciculus retroflexus; G, granule cell layer of the cerebellar cortex; h, hilus; M, molecular layer of the cerebellar cortex; opt, optic tract; Th, thalamus. Bars: 50 μ m in B, E, and F; 500 μ m in C and D.



gave a strong signal in the developing and adult nervous tissues, whereas the sense probe did not give any clear signal (unpublished data). A clear AMIGO expression was already detected in the E13 rodent embryo; at this stage the highest expression level was found in the dorsal root ganglia and the trigeminal ganglion, with some expression in the central nervous system (Fig. 3 B, a and b). During later stages of development and in the adult, AMIGO was also prominently expressed in the brain, where the most intense signal was detected in the hippocampus (Fig. 3 B, c).

To investigate the expression of AMIGO at the protein level, polyclonal antisera were produced against an extracellular 10-amino acid peptide sequence that is found in AMIGO, but not in AMIGO2 or AMIGO3. The anti-peptide antibodies recognized the 75-kD AMIGO–Ig fusion protein produced in *Drosophila* S2 cells (Fig. 4 A, lanes 1 and 3). Western blotting of crude brain extracts revealed specific binding to a 65-kD polypeptide (Fig. 4 A, lanes 2 and 4). The molecular mass of the recognized polypeptide is close to the calculated molecular mass (56 kD) of AMIGO. Binding of the antibodies to both the fusion protein and the 65-kD polypeptide of brain were blocked by the synthetic peptide used as the immunogen (Fig. 4 A, lanes 3–6). Furthermore, binding of the antibodies to tissue sections was blocked by the synthetic peptide (Fig. 4 B).

Western blotting of AMIGO using crude brain extracts from different developmental stages was consistent with the

in situ hybridization data. The expression appears to start in the brain somewhat later than in the peripheral nervous system, and increases clearly between E13 to E14 (Fig. 4 C). The expression is maintained high during the perinatal developmental stage, but is down-regulated during the postnatal stages P6 to P10. After this, the expression is again up-regulated and remains high in the adult brain (Fig. 4 C). Because the time period of the postnatal up-regulation of the AMIGO expression appears to coincide with the onset of myelination, we compared the expression of AMIGO to that of the myelin-specific marker α -CNPase. Indeed, the expressions of AMIGO and the CNPase display a parallel increase during postnatal development (Fig. 4 C). Thus, the AMIGO expression displays a dual character during brain development; the first expression peak occurs during the late embryonic and perinatal development, and the second increase in expression accompanies myelination.

Immunohistochemistry using the anti-peptide antibodies revealed specific staining only in the nervous system. Intensity of the immunostaining was in agreement with the expression data inferred from Western blotting (Fig. 4 C), and specificity of the immunostaining was also suggested by inhibition of antibody binding to AMIGO and to tissue sections by the peptide used as the immunogen (Fig. 4, A and B). In general, AMIGO was intensely stained in developing and mature fiber tracts (Fig. 5, A–F). During embryonic develop-

ment, when the spinal ganglia abundantly express AMIGO mRNA (see Fig. 3 B, a and b), the immunostaining was observed in the fiber tracts connecting to the ganglia and the spinal cord, but not in the ganglia themselves (Fig. 5 E), suggesting that the AMIGO protein is transported to axonal processes. In cerebellum, the most intense staining was observed in fibers on both sides of the Purkinje cell layer; the characteristic structure formed by the basket cell axons around the Purkinje cell soma was clearly discerned by the AMIGO immunostaining (Fig. 5 B). Consistent with the Western blotting data, AMIGO immunostaining labeled most myelinated axon tracts in the adult (Fig. 5 A). An example is shown in Fig. 5 (C and D), demonstrating the similarity of the AMIGO and α -CNPase immunostaining around the hippocampus. However, the AMIGO expression is not restricted to myelinated tracts; for example, in hippocampus, nonmyelinated tracts in the stratum lucidum CA3 region, which were negative for α -CNPase (Fig. 5 D) and myelin basic protein (unpublished data), stained clearly for AMIGO (Fig. 5 C). In general, AMIGO staining was detected (both during development and in adult animal) in large-diameter neurites (axons) that were also stained by antibodies against the 145-kD neurofilament (unpublished data). As in the forebrain, myelinated axon tracts were also stained for AMIGO in cerebellum, pons, medulla, and spinal cord.

AMIGO was also clearly immunostained both in the cell soma and in fasciculated and nonfasciculated processes of cultured hippocampal neurons (Fig. 5 F). As expected from immunostaining of tissue sections, double-immunostaining (unpublished data) revealed colocalization with the 145-kD

neurofilament and the β -tubulin (TuJ1), but not with MAP2. Thus, AMIGO is preferentially expressed in axonal rather than dendritic processes.

AMIGO promotes neurite extension of hippocampal neurons

Identification of AMIGO from hippocampal neurons growing neurites on amphoterin, the occurrence in fiber tracts *in vivo*, and the domain structure with LRRs and Ig domains suggest that AMIGO might have a role in neurite extension. To get insight into the function of AMIGO, we tested if it is able to promote neurite outgrowth of hippocampal neurons. The extracellular part of the AMIGO was fused to human IgG Fc part, and this fusion protein was immobilized on microtiter wells and used as a substrate for hippocampal neurons. These experiments showed that the AMIGO-Ig fusion protein promotes attachment and neurite outgrowth of hippocampal neurons (Fig. 6, A and C), whereas on the human IgG Fc control, neurite outgrowth was very low or undetectable (Fig. 6, B and C). Neurite outgrowth induced by the immobilized AMIGO-Ig fusion protein was inhibited by the soluble AMIGO-Ig fusion in the culture medium (Fig. 6 D).

Soluble AMIGO perturbs development of fasciculated axon tracts *in vitro*

Because AMIGO immunostaining could be found *in vitro* in hippocampal fasciculating axons and in the axon tracts *in vivo*, AMIGO might participate in fasciculation of neurites. We addressed this question by using the ectodomain of

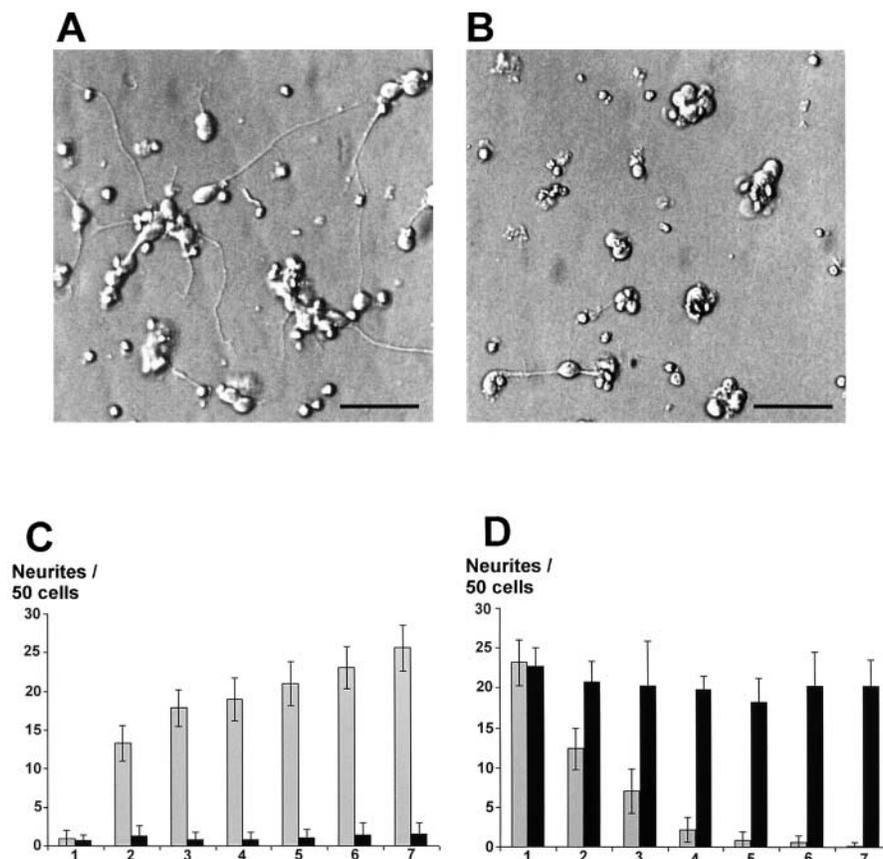


Figure 6. AMIGO promotes neurite outgrowth of hippocampal neurons. (A) Substrate coated with 25 μ g/ml of AMIGO promotes neurite outgrowth of E18 hippocampal neurons. (B) Cells on the control substrate coated with 25 μ g/ml of the Fc protein without the AMIGO ectodomain is shown for comparison. (C) AMIGO induced hippocampal neurite outgrowth after 24 h of culture. The AMIGO-Ig fusion (gray bars) and the Fc control protein (black bars) were coated as follows: (1) 0 μ g/ml, (2) 3.125 μ g/ml, (3) 6.25 μ g/ml, (4) 12.5 μ g/ml, (5) 25 μ g/ml, (6) 50 μ g/ml, and (7) 100 μ g/ml. (D) AMIGO-induced neurite outgrowth (the substrate coated with 25 μ g/ml of the AMIGO-Ig fusion protein) is blocked by the AMIGO-Ig fusion protein in the assay medium. The AMIGO-Ig fusion (gray bars) and the Fc control protein (black bars) were added into the culture medium as follows: (1) 0 μ g/ml, (2) 3.125 μ g/ml, (3) 6.25 μ g/ml, (4) 12.5 μ g/ml, (5) 25 μ g/ml, (6) 50 μ g/ml, (7) and 100 μ g/ml. The error bars give the SD calculated from 15 microscopy fields in three independent experiments. Bar, 50 μ m in A and B.

AMIGO as Ig fusion protein in the culture medium. Hippocampal neurons were plated on poly-L-lysine-coated wells to promote neurite outgrowth and fasciculation. Microscopy of the cultures revealed that the growth pattern of neurites was dramatically changed in the presence of the soluble AMIGO. In the control cultures, neurites formed fascicles in 4 d, whereas in the presence of the soluble AMIGO, the processes were mainly nonfasciculated up to at least 5 d in culture (Fig. 7, A–C).

AMIGO, AMIGO2, and AMIGO3 display a homophilic and heterophilic binding mechanism

Fasciculation of axons is known to involve homophilic interactions, and this might be reason why soluble AMIGO perturbs fasciculation. Therefore, we tested in a coimmunoprecipitation assay whether AMIGO, AMIGO2, and AMIGO3 could bind themselves and whether the AMIGOs display heterophilic binding within the protein family. To examine homo- and heterophilic association, 293T cells were cotransfected with GFP-tagged AMIGOs, V5-tagged full-length AMIGOs, and the control protein V5-tagged RAGE (Fig. 8 A). Immunoprecipitation of the GFP-tagged AMIGOs from the cell lysates precipitated all V5-tagged AMIGOs, and correspondingly, the GFP-tagged AMIGOs were all precipitated with anti-V5 antibodies (Fig. 8 A). The control protein V5-tagged RAGE was not coprecipitated with the AMIGO-, AMIGO2-, or AMIGO3-GFP and vice versa. (Fig. 8 A).

As another approach to study homophilic binding of the AMIGOs, we added AMIGO-, AMIGO2-, and AMIGO3-Ig fusion protein to protein A-coated beads to get the protein oriented in a manner that occurs at the cell surface. AMIGO and AMIGO2 caused rapid aggregation of the beads (Fig. 8 B and Fig. 8 C, b and c), but for AMIGO3 the aggregation was milder (Fig. 8 B and Fig. 8 C, d). Addition of the IgG Fc part as the control protein did not induce any clear aggregation (Fig. 8 B and Fig. 8 C, a).

Discussion

A novel family of transmembrane proteins with six LRR domains and one Ig-like domain

In this paper, we have identified a novel family of transmembrane proteins called AMIGO, AMIGO2, and AMIGO3. These three proteins show clear homology with each other; their length and domain organization are highly identical (Fig. 2 B). This domain relationship suggests a common evolutionary origin of the AMIGOs.

Based on genomic sequence data, these three proteins probably occur in the puffer fish *Fugu rubripes* (unpublished data). Interestingly, *Drosophila* has a protein family called kekkon with three members of transmembrane proteins, kek1, kek2 (Musacchio and Perrimon, 1996), and kek3 (Ashburner et al., 1999), which show homology in their extracellular parts to the AMIGOs. The extracellular parts of both the AMIGOs and the kek proteins contain six LRR domains flanked with cysteine-rich LRRNT and LRRCT domains and one Ig domain close to the transmembrane region. However, the cytoplasmic parts of the AMIGOs and kek proteins do not display homology with each other. The

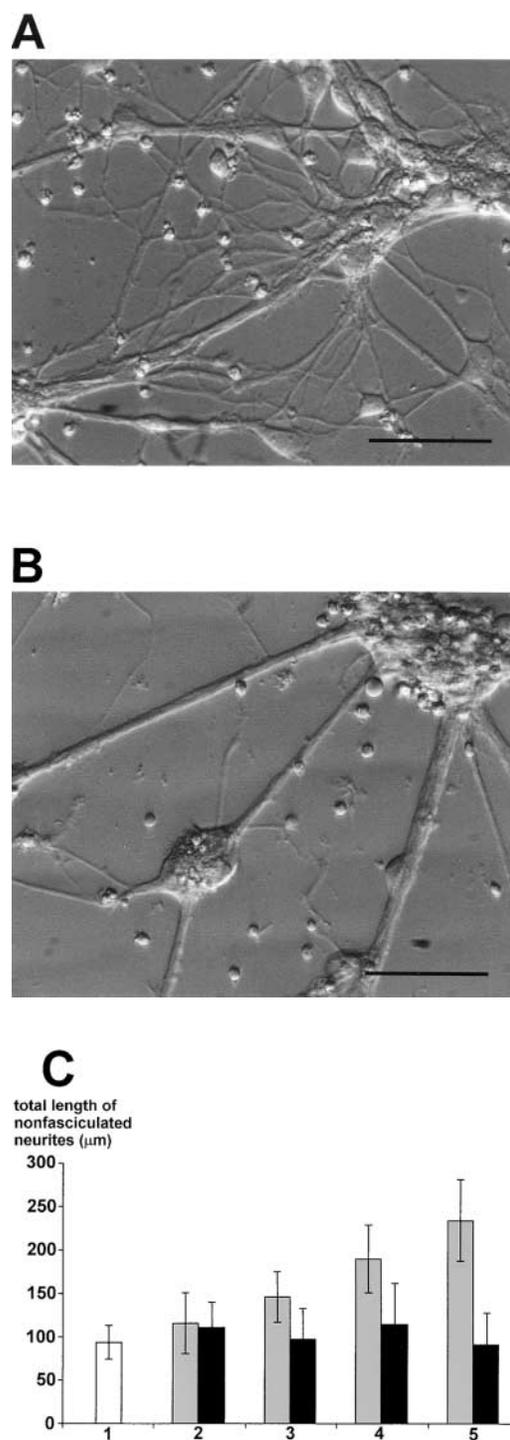


Figure 7. **Soluble AMIGO inhibits fasciculation in hippocampal neurons.** (A) E18 hippocampal neurons on poly-L-lysine substrate with 25 µg/ml of the AMIGO-Ig fusion in the culture medium. (B) E18 hippocampal neurons on poly-L-lysine substrate with 25 µg/ml the Fc control protein is shown for comparison. (C) Total length of processes, the diameter of which is <2 µm (formed from 1–3 neurites) on poly-L-lysine substrate. The AMIGO-Ig fusion and the Fc control protein (black bars) were added into the culture medium as follows; (1) 0 µg/ml (white bar), (2) 3.125 µg/ml, (3) 6.25 µg/ml, (4) 12.5 µg/ml, and (5) 25 µg/ml. The error bars in C give the SD calculated from 12 microscopy fields in three independent experiments. Bar, 50 µm in A and B.

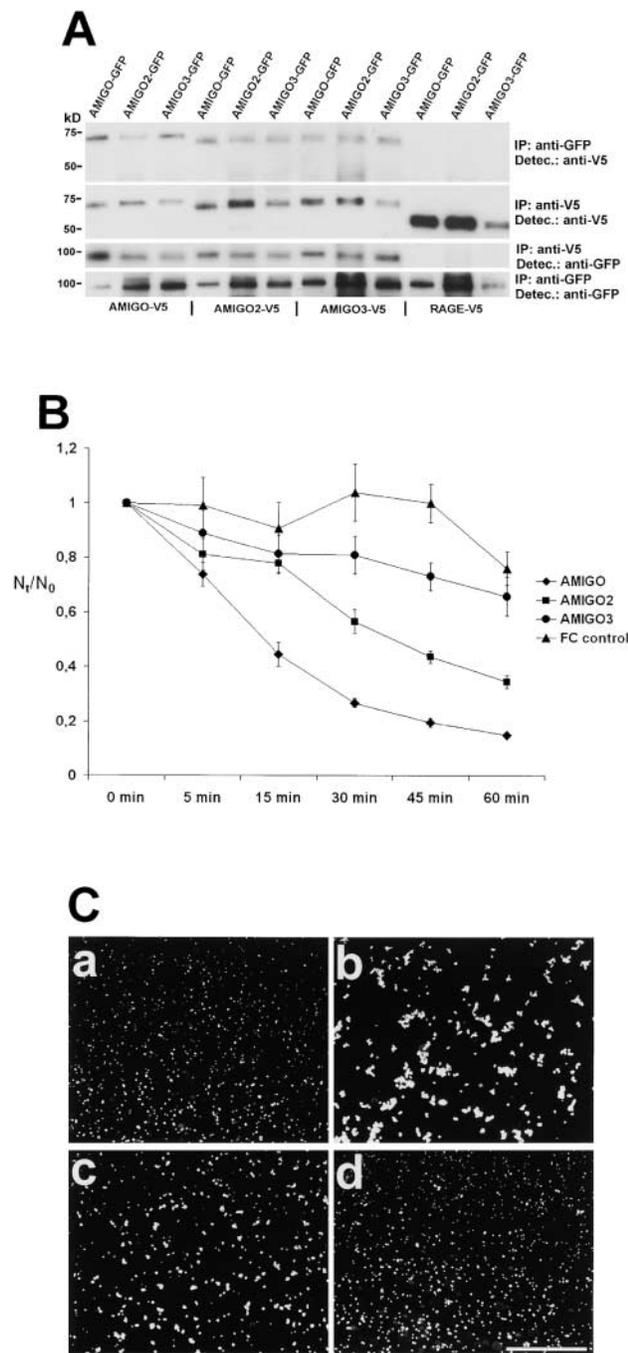


Figure 8. Homophilic and heterophilic interaction of the AMIGO family members. (A) Co-immunoprecipitation experiment. Cells were double transfected with different AMIGO or RAGE constructs (as indicated on top and bottom of the figure). Full-length V5-tagged AMIGO, AMIGO2, and AMIGO3 were coprecipitated with full-length GFP-tagged AMIGO, AMIGO2, and AMIGO3, whereas the V5-tagged RAGE was not coprecipitated with any of the three GFP-tagged AMIGOs. Coprecipitation could be also shown by precipitating with V5 antibody. (B) Kinetics of bead aggregation. N_t and N_0 are the total number of particles at incubation times t and 0, respectively. The extent of bead aggregation is represented by the index N_t/N_0 . Diamond curve (◆) represents AMIGO-Ig fusion coated beads, square curve (■) AMIGO2, sphere curve (●) AMIGO3 and triangle curve (▲) represents Fc control protein coated beads. The error bars give the SD calculated from eight microscopy fields in two independent experiments. (C) Bead aggregation after 60 min using

gene expression data of *kek1* and *kek2* (Musacchio and Perimon, 1996) is reminiscent of the one seen for AMIGO and AMIGO2; they all are expressed in the central nervous system of the adult organism. These domain and expression similarities suggest that the AMIGOs and *kek* proteins may be derived from a common ancestral gene.

In their extracellular parts, the most homologous motifs between the AMIGOs are the LRRs 3–5. The best fit in BLAST searches shows homology with Slit family of extracellular axon-guiding proteins (Whitford et al., 2002), and a clear homology is also found with the Nogo-66 receptor, where the only recognizable motifs are the LRR domains (Fournier et al., 2001; Fig. 2 C). The similarity found in the LRRs in AMIGO, Slit1, and Nogo-66 receptor suggests an evolutionary origin of these proteins from a common ancestor. The clear conservation seen at the LRR area between the AMIGOs suggests that this region is important for interactions with extracellular ligand(s), and that they could also share the same binding partner(s).

In the literature, there are reports of other transmembrane proteins that contain LRRs and Ig domains in the extracellular part of the proteins. ISLR (Nagasawa et al., 1997), 5 LRRs and 1 Ig domain; Pal (Gomi et al., 2000), 5 LRRs and 1 Ig domain; LIG-1 (Suzuki et al., 1996), 15 LRRs and three Ig domains; and GAC1 (Almeida et al., 1998), 12 LRRs and 1 Ig domain. Common for all of these proteins and the AMIGOs is the order of how the LRRs and the Ig domains are organized; the LRRs are always more distal to the transmembrane region than the Ig domains. Interestingly, BLAST searches by using Ig domain sequences from AMIGOs give no distinct homology with other Ig domains of the Ig superfamily proteins, but the closest are the ones found in proteins containing both Ig and LRR domains (unpublished data).

Although the cytoplasmic moieties of the AMIGOs do not display any significant homology with previously identified transmembrane proteins, the alignment of the AMIGOs (Fig. 2 A) shows two conserved serine-rich regions; one close to the transmembrane domain and the other at the COOH terminus. The COOH-terminal serine-rich area of AMIGO and AMIGO2 has a consensus sequence for Casein kinase II serine/threonine kinase (Allende and Allende, 1995), which is ubiquitously expressed in brain, but AMIGO3, which is not expressed in the brain, does not have this consensus sequence. Recently, Watts et al. (1999) showed that the transmembrane form of TNF- α has a consensus sequence SXXXS that is a substrate for Casein kinase I-dependent phosphorylation. Interestingly, all three AMIGOs have four possible Casein kinase I phosphorylation sites in these two conserved serine-rich areas. Future work will reveal whether these conserved serine residues have important functions in signaling events of the AMIGOs.

There are an increasing number of reports in the literature and the data banks on mammalian transmembrane proteins having both LRR and Ig domains, but unfortunately at

protein A beads coated with the Fc control protein (C, a), the AMIGO-Ig fusion (C, b), AMIGO2-Ig fusion (C, c) or the AMIGO3-Ig fusion protein (C, d). Bar, 100 μ m for a–d.

present, almost all data only comprise the cloning and tissue expression of these proteins. Our data here give a functional insight into these twin motif transmembrane proteins, belonging to both the LRR and Ig superfamily, in a form of more detailed characterization of AMIGO.

AMIGO, a novel transmembrane protein in neuronal processes with homophilic binding mechanism

Based on RT-PCR experiments, *in situ* hybridization, and immunohistochemistry, AMIGO is essentially a nervous system-specific protein. Interestingly, AMIGO expression is up-regulated at two clearly distinct stages during brain development; the first peak is found perinatally, and the second up-regulation occurs during or slightly before the up-regulation of the oligodendrocyte-specific marker α -CNPase.

The first expression peak of AMIGO is compatible with a role in growth of axonal connections. The expression of AMIGO in developing axon tracts both *in vivo* and *in vitro* and our neurite outgrowth experiments support this role. One cellular mechanism in the growth of axonal connections is fasciculation. Axons grow along each other by using pioneer axons as the substratum for the growth cones of the later ones. Interestingly, addition of AMIGO ectodomain in the culture medium inhibits the formation of neurite bundles, suggesting a role for AMIGO in fasciculation. Furthermore, AMIGO displays a homophilic binding mechanism that would explain its role in fasciculation. Homophilic adhesion molecules belonging to both the Ig superfamily and to the cadherin family have been shown to mediate neurite outgrowth and fasciculation during the nervous system development (for review see Kamiguchi and Lemmon, 1997; Martinek and Gaul, 1997). It is also noteworthy that the LRR sequences of the AMIGOs display homology to the Slit proteins and to the Nogo receptor (Fig. 2 C) that have been implicated in axon growth, regeneration, and guidance. Further studies are thus warranted to identify ligands of AMIGO that do not belong to the AMIGO family and to study possible association of AMIGO to other transmembrane proteins.

The second up-regulation of the AMIGO expression suggests a role in myelination. It seems reasonable that AMIGO would mediate cell-cell interactions also at this stage of development. However, further studies are clearly warranted to understand the role of AMIGO in myelinating axon tracts, like in the interactions of axons with oligodendrocytes and Schwann cells. Interestingly, our present results suggest that the members of the AMIGO family are able to bind each other in a heterophilic fashion in addition to homophilic binding detected in all family members (Fig. 8, A–C). It is tempting to speculate that AMIGO and AMIGO2, which both are expressed in the central nervous system (Fig. 3 A), have a cooperative role by binding each other from opposing cell membranes, for example, in contacts of axons with each other or with glial cells.

Furthermore, AMIGO expression remains high until adulthood. This suggests that AMIGO plays a role in regeneration and plasticity of the adult fiber tracts, the mechanisms of which commonly recapitulate mechanisms of fiber tract development. From this viewpoint, it is of interest that AMIGO expression is induced by ligation of the Ig superfamily protein RAGE by amphoterin and by anti-RAGE, whereas integrin ligation by laminin does not induce

AMIGO expression (Fig. 1, B and C). In general, RAGE signaling plays a role in tissue injury and in regulation of cell motility, for example, in growth cone and cell migration (for review see Rauvala et al., 2000; Schmidt et al., 2000).

To get further insight into the functional roles of AMIGO during development and adulthood, we have recently targeted the gene in ES cells and are currently producing AMIGO-null mice (unpublished data). In addition to the *in vivo* approaches using gene targeting, it will be important to understand what molecular domains mediate homophilic and heterophilic binding of the AMIGO family, and whether the intracellular domains of the AMIGOs have signaling properties. Furthermore, future studies will reveal whether the members of the AMIGO family mediate analogous cell-cell interactions in nonneuronal and neuronal tissues characterized in the present paper for AMIGO in axonal tracts.

Materials and methods

ODD

ODD was performed as described by Matz et al. (1997), comparing genes induced on amphoterin versus laminin matrix. Hippocampi were dissected from 18-d-old rat embryos and triturated with a Pasteur pipette in HBSS (without calcium and magnesium; GIBCO BRL) containing 1 mM sodium pyruvate and 10 mM Hepes, pH 7.4. After washing in HBSS, neurons were suspended in neurobasal medium (GIBCO BRL), 2% B27 supplement (GIBCO BRL), 25 μ M L-glutamic acid (Sigma-Aldrich), and 1% L-glutamine (GIBCO BRL), and were then seeded at the density of 10^6 cells on 35-mm plastic plates (Greiner Bio-One) coated with 10 μ g/ml laminin (Sigma-Aldrich) or 10 μ g/ml recombinant amphoterin. RNA was isolated by using the RNeasy mini kit (QIAGEN) 24 h after seeding, and was used for ODD.

Cloning of the AMIGO, AMIGO2, and AMIGO3 cDNAs

The rat AMIGO cDNA 5' end was amplified using the method of Matz et al. (1999), based on template-switching effect and step-out PCR, and the full-length cDNA was cloned from postnatal d 14 rat cerebrum using a reverse transcription reaction with a 5' primer (5'-ACTGCTTCTCGCCTGCCCCG-3') and a 3' primer (5'-GAACCTCCCCATCAGCCTATACTG-3'). The rat AMIGO sequence was used to find out human and mouse ESTs to get sequences for cloning of the human and mouse AMIGOs. The human AMIGO cDNA was cloned from the THP-1 cell line (TIB-202; American Type Culture Collection) using a reverse transcription reaction with a 5' primer (5'-CAGAACATGCCCGGGTGAC-3') and a 3' primer (5'-GGACCAATTCCTTGAGGTGAC-3'). The mouse AMIGO cDNA was cloned from adult mouse cerebrum using a reverse transcription reaction with a 5' primer (5'-ACTGCTTCTCGCCTGGCCCCG-3') and a 3' primer (5'-AACCTCCCCATCAGCCTACGCTG-3'). The AMIGO sequences were used for homology search with BLAST to find other possible related sequences. The human AMIGO2 cDNA was cloned from the HT1080 cell line (CCL-121; American Type Culture Collection) as above: the 5' primer was 5'-CTCAGAGCGCATAATGTC-3', and the 3' primer was 5'-TGTTTATTTG-CAGACCACACAC-3'. The mouse AMIGO2 cDNA was cloned from adult mouse cerebrum with a 5' primer (5'-CTCAGAGCGCATAATGTC-3') and a 3' primer (5'-GCGATGCTGAAGGCTAAGATG-3'). The human AMIGO3 cDNA was cloned from the HEK293 cell line (CRL-1573; American Type Culture Collection) with a 5' primer (5'-CAACCTGCACAGAGCTGCTGTGAC-3') and a 3' primer (5'-GCACAGTGCTCCAC-CAGTATCTG-3'). The mouse AMIGO3 cDNA was cloned from adult mouse cerebellum with a 5' primer (5'-AGAAGTAGTGAGTCTTG-GAGCT-3') and a 3' primer (5'-TGTTGTGAGGTAGAGCCTG-3').

RT-PCR and *in situ* hybridization

Total RNA was reverse transcribed in a reaction containing 1 μ g RNA, 0.25 mM dNTP-mix, 1 μ g random nonamers, 20 U recombinant RNasin[®] (Promega), 200 U MMLV-RT (Promega) with 1 \times MMLV reaction buffer supplied. 2 μ l of the reverse transcription mixture was then used for polymerase chain reaction with gene-specific primers. For the mouse and rat AMIGO, the primers were a 5' primer (5'-AGCAACATCCTCAGCTGCTC-3') and a 3' primer (5'-CTCAGCTTGTGGAGGACAG-3'). For mouse AMIGO2, primers were a 5' primer (5'-GGCACTTTAGCTCCGTGATG-3') and a 3' primer (5'-GTCTCGTTTAAACAGCCGCTG-3'). For the mouse

AMIGO3, primers were a 5' primer (5'-AGGTGTACAGTCCCGAGTG-3') and a 3' primer (5'-GTAGAGCAACACCAGCACCA-3'). For rat ChrB, the primers were a 5' primer (5'-CTTCCCAGAGTACAACACTATGAC-3') and a 3' primer (5'-GCTGAACCTTCCGCTATCTTC-3'). For GAPDH control, primers were a 5' primer (5'-CAACGACCCCTTCATTGACC-3') and a 3' primer (5'-AGTGATGGCATGGACTGTGG-3').

The subsequent PCR reaction was performed in a PCR mix (2.5 μ M dNTP, 10 mM Tris-HCl, pH 8.8, 150 mM KCl, 1.5 mM MgCl₂, and 0.1% Triton X-100) containing 0.2 μ M 5' primer and 3' primer and 1 U DynaZyme™ II DNA Polymerase (Finzymes). The amplification products were separated on 1.5% agarose gel and stained with EtBr.

For in situ hybridization with radiolabeled probes, a 1.2-kb fragment from the mouse AMIGO cDNA was PCR amplified with a 5' primer (5'-CCGCTC-GAGCCGGCCGATCTGTGGTTAG-3') and a 3' primer (5'-CGGAATCTC-CACACCACAATGGTCTATCAGA-3'). The reaction product was then ligated into pGEM-T vector. In situ hybridization analysis was performed using single-stranded RNA probes on mouse fetal and adult paraffin-embedded tissue sections as described previously (Reponen et al., 1994).

Production of AMIGO, AMIGO2, and AMIGO3-Ig fusion proteins

A 1,180-bp BamHI fragment containing the entire extracellular coding region of the mouse AMIGO was amplified by PCR with a 5' primer (5'-CGGGATCCTAGGGTACTCTCTCCAGATCC-3') and a 3' primer (5'-CGGGATCCGTTGAGGGTTCATGGTGTCC-3'). The reaction product was then ligated into pRHA3-3c-Fc-cDNA. The AMIGO2 and AMIGO3 extracellular coding region without signal sequence was cloned into the modified pRHA3 vector, which contains CD33 signal sequence and Fc-cDNA from signal plgplus vector. A 1,065-bp NheI/NotI fragment of the mouse AMIGO2 was amplified with a 5' primer (5'-TACTAGCTAGCTGCC-CCACTGCTTGCATCTG-3') and a 3' primer (5'-ATAGTTAGCGGC-CGCTCGTGGGCGTGGGA-3'). A 1,080-bp NheI/NotI fragment of the mouse AMIGO3 was amplified with a 5' primer (5'-CTATACTAGCTAGC-GATGTTCTGCCTCTGCTCC-3') and a 3' primer (5'-ATAGTTAGCG-CGCTCGTGGGAAAGTCTCTGGCTCG-3').

The AMIGO, AMIGO2, and AMIGO3-Ig fusion protein plasmids were cotransfected with the hygromycin resistance plasmid p-COP-hyg into *Drosophila* S2 cells using the FuGENE™6 transfection reagent (Roche). After a 3-wk selection with 300 μ g/ml hygromycin B (Calbiochem), stable AMIGO-Ig fusion S2 cell pools were cultured in shake flasks, where the protein expression was induced with 500 μ M CuSO₄. After culturing for 6 d, the AMIGO-Ig fusion proteins were isolated from the supernatant by using protein A-agarose (Upstate Biotechnology) according to the manufacturer's instructions.

Antibodies, Western blotting, and immunohistochemistry

Rabbit anti-AMIGO peptide antibodies were raised against the synthetic peptide YAMGETFNET (corresponding to amino acids 341–350 of the mouse AMIGO and 342–351 of the rat and human AMIGO). Binding of the antibodies to AMIGO was verified using the recombinant AMIGO-Ig fusion protein and crude brain extracts in Western blotting (see below). Because the antibodies bound more intensely and specifically to the rat AMIGO compared with AMIGO from other species (possibly due to species differences in the glycosylation site close to the peptide sequence used in immunization), rat samples were primarily used in immunochemical detections.

Rabbit anti-RAGE peptide antibodies were raised against the synthetic peptide NTGRTEAWKVLSPQG (corresponding to amino acids 54–68 of the mouse, rat, bovine, and human RAGE). The RAGE antibodies were affinity-purified as described previously (Rauvala et al., 1988) by using the same peptide used for immunization. The synthetic control peptide, which was used to validate the specificity of the anti-RAGE antibodies, have the sequence RPLNTAPIQPRVRE (corresponding to amino acids 217–231 of mouse and 216–230 of rat RAGE).

Brains of embryonic, postnatal, and adult rats were extracted to the final concentration of 83.3 mg tissue/ml SDS extraction buffer (62.5 mM Tris, 1.8% SDS, 7.75% glycerol, and 4.4% 2-mercaptoethanol, pH 6.8). After addition of the SDS buffer, the extracts were pressed several times through a needle. The extracts were boiled 2 \times 5 min and centrifuged at 10,000 g for 10 min to remove nonsoluble material. Samples corresponding to the same wet weight of tissue were analyzed by Western blotting. Ponceau staining of the membrane confirmed uniform protein amounts.

Precast 4–15% gels (Bio-Rad Laboratories) were used for SDS-PAGE in Western blotting. Proteins were transferred to Hybond™ nitrocellulose membrane (Amersham Biosciences) by semi-dry blotting technique. Rabbit anti-AMIGO peptide antibody (1/1,000 dilution) and monoclonal anti-CNPase, clone 11–5B (1/1500; Sigma-Aldrich) were used as primary antibodies.

HRP-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) and sheep anti-mouse IgG (Amersham Biosciences) were used as secondary antibodies. The antibody complexes were detected using ECL™ reagents (Amersham Biosciences).

Immunohistochemistry of AMIGO was performed using paraffin sections. In brief, adult rats were killed after CO₂ treatment by cervical dislocation, tissues were fixed using ice-cold PBS with 4% PFA, and the samples were then transferred in paraffin. Hydrated paraffin sections (4–10- μ m thick) were incubated with 1% hydrogen peroxide/methanol solution for 20 min, and were washed again with PBS. The sections were blocked for 1 h with 5% skimmed milk powder in PBS. The sections were then incubated with the rabbit AMIGO peptide antiserum, which was diluted 1/200 in the blocking buffer at 4°C overnight. After washing with PBS, the sections were incubated with HRP-conjugated goat anti-rabbit antibodies (Bio-Rad Laboratories) at a dilution of 1:500 for 2 h at RT, washed with PBS, and incubated with aminoethyl carbazole (Sigma-Aldrich) as a chromogenic substrate. Immunofluorescence staining for in vitro cultured hippocampal neurons was performed by using FITC-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories).

Neurite outgrowth assay

Hippocampi were dissected from 18-d-old rat embryos into a calcium/magnesium-free trituration medium (HBSS with 1 mM sodium pyruvate and 10 mM Hepes, pH 7.4). Cells were dissociated by pipetting 25 times with a glass Pasteur pipette and were washed once with the calcium/magnesium-containing buffer (HBSS plus calcium and magnesium with 1 mM sodium pyruvate and 10 mM Hepes, pH 7.4). The cells were seeded at the density of 70,000 cells/cm² on 96-well polystyrene dishes coated by the test protein in Neurobasal medium with 2% B27 supplement (GIBCO BRL), 1% BSA, 0.5 mM L-glutamine, 25 μ M L-glutamic acid, and 1 \times penicillin-streptomycin. The dishes were coated with the test protein (3.125–100 μ g/ml) in PBS overnight at 4°C, washed three times with PBS, and blocked with 1% BSA in PBS for 1 h at RT before adding the cells. The cells were cultured for 24 h before counting the neurite outgrowth. For counting of neurite outgrowth, images were taken from living cells using randomly selected microscopic fields, and the extensions, which were twice the length of the cell soma, were considered as neurites. For quantification of neurite outgrowth, 15 images (275 \times 225 μ m) with a total of 750 cells were evaluated from every concentration of the test protein (AMIGO-Ig fusion or Fc control substrate) used for coating. The data were pooled from three independent experiments.

To test the effect of soluble AMIGO-Ig fusion protein, the dishes were coated with the AMIGO-Ig fusion protein (12.5 μ g/ml in PBS) at 4°C overnight, washed three times with PBS, and blocked with 1% BSA in PBS for 1 h at RT. The cells were seeded at the density of 70,000 cells/cm² and cultured for 24 h before counting the neurite outgrowth. Counting was performed as above from three independent experiments. A total of 750 cells were evaluated for every concentration of the test protein (AMIGO-Ig fusion or the Fc control protein) used in solution.

In vitro fasciculation assay

Fasciculation of neurites was studied with hippocampal neurons prepared as above. The 96-well plates were coated with poly-L-lysine at 4°C overnight, washed three times with PBS, and blocked with 1% BSA in PBS for 1 h at RT. The cells were seeded at the density of 70,000 cells/cm² in the serum-free medium (see Neurite outgrowth assay) with either the AMIGO-Ig fusion protein or the Fc control protein in solution. The AMIGO-Ig fusion and the Fc control protein were tested at 3.25–25 μ g/ml. The experiment was repeated independently three times, and pictures were taken from living cells after 4 d in culture. For quantification of neurite outgrowth, 12 randomly taken images (45 \times 35 μ m) were taken for every concentration of the AMIGO-Ig fusion and the Fc control protein used in solution. To evaluate inhibition of fasciculation, the total length of the processes, the diameter of which is < 2 μ m (formed only from 1–3 neurites), was measured from the 12 images taken for every protein concentration tested.

Pictures for the neurite outgrowth and fasciculation experiments were taken with a digital camera (model DP10; Olympus). The measurements were performed using the Image-Pro® image analysis software (Media Cybernetics, Inc.).

Binding assays

Coimmunoprecipitation experiments were performed using transiently transfected HEK293T cells. The constructs were transfected into the cells using FuGENE™6 (Roche) according to the manufacturer's instructions. The full-length AMIGO, AMIGO2, and AMIGO3 were cloned in frame with the pEGFP-N1 (CLONTECH Laboratories, Inc.) and pcDNA6-V5-His (Invitro-

gen) vectors. Full-length RAGE was cloned in frame with the pcDNA6-V5-His vector. After transfection, the cells were grown for 48 h before lysing in the RIPA buffer with 10 mg/ml PMSF and 60 μ g/ml aprotinin (Sigma-Aldrich). Coimmunoprecipitation experiments were performed using rabbit anti-GFP antibody (sc-8334; Santa Cruz Biotechnology, Inc.) and mouse anti-V5 antibody (46-0705; Invitrogen) at the concentration of 1 μ g/ml.

The aggregation assay was performed using 1- μ m protein A Fluoresbrite™ carboxylated beads (Polysciences, Inc.). The 100- μ g beads were first washed three times with PBS, 2% BSA, and 0.1% Tween 20 solution, and were then mixed and sonicated in water bath in 50 μ l of the buffer mentioned above. The beads were divided to two aliquots, and the test and the control protein (10 μ g each) were added into the beads in 25 μ l of PBS, 2% BSA, and 0.1% Tween 20 solution (final volume 50 μ l). The aggregation samples were incubated at RT. After addition of the protein, 1.5- μ l samples were taken into 100 μ l of PBS, 2% BSA, and 0.1% Tween 20 solution in 96-well plates at different time points. The aggregation was evaluated and filmed under the fluorescence microscope. Kinetics of bead aggregation was calculated from two independent experiments from eight fields containing 9,000 beads. The extent of bead aggregation is represented by the index N_t/N_0 , where N_t and N_0 are the total number of particles at the incubation times t and 0 (Agarwala et al., 2001).

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