

Identification of a novel contactin-associated transmembrane receptor with multiple domains implicated in protein–protein interactions

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Receptor protein tyrosine phosphatase β (RPTP β) expressed on the surface of glial cells binds to the glycosylphosphatidylinositol (GPI)-anchored recognition molecule contactin on neuronal cells leading to neurite outgrowth. We describe the cloning of a novel contactin-associated transmembrane receptor (p190/Caspr) containing a mosaic of domains implicated in protein–protein interactions. The extracellular domain of Caspr contains a neurophilin/coagulation factor homology domain, a region related to fibrinogen β/γ , epidermal growth factor-like repeats, neurexin motifs as well as unique PGY repeats found in a molluscan adhesive protein. The cytoplasmic domain of Caspr contains a proline-rich sequence capable of binding to a subclass of SH3 domains of signaling molecules. Caspr and contactin exist as a complex in rat brain and are bound to each other by means of lateral (*cis*) interactions in the plasma membrane. We propose that Caspr may function as a signaling component of contactin, enabling recruitment and activation of intracellular signaling pathways in neurons. The binding of RPTP β to the contactin–Caspr complex could provide a mechanism for cell–cell communication between glial cells and neurons during development.
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Introduction

A large class of protein tyrosine phosphatases exhibits structural features common to cell surface receptors. These receptor-like protein tyrosine phosphatases (RPTPs) are composed of an extracellular domain, a single transmembrane domain and a cytoplasmic portion that contains one or two tyrosine phosphatase domains (Fisher *et al.*, 1991; Walton and Dixon, 1993). The extracellular domains of several RPTPs have hallmarks typical of adhesion molecules, suggesting that these receptors may play a role in cell–cell communication (Brady-Kalnay and Tonks, 1995). RPTP β (also known as PTP ζ) and RPTP γ are two members of a subfamily of RPTPs that contain in their extracellular domain a region with sequence homology to the enzyme carbonic anhydrase (CAH). In both RPTP β

and RPTP γ , the CAH domains are followed by a fibronectin type III (FNIII) repeat and by a long unique sequence termed the spacer domain (Krueger and Saito, 1992; Barnea *et al.*, 1993; Levy *et al.*, 1993). Three different isoforms of RPTP β are expressed as a result of alternative RNA splicing; a short and a long receptor form that differ by the presence of a stretch of 860 amino acid residues in the spacer domain, and a secreted form, composed of only the extracellular domain of RPTP β , also known as the 3F8 proteoglycan or phosphacan (Levy *et al.*, 1993; Barnea *et al.*, 1994; Maurel *et al.*, 1994). It has been shown that the expression of RPTP β is restricted to the nervous system. RPTP β is expressed in cells that have been implicated in neuronal migration and axonal guidance, including glial precursors, radial glia and astrocytes (Rauch *et al.*, 1991; Canoll *et al.*, 1993). RPTP β also bears the HNK-1 carbohydrate epitope that is found in several neuronal adhesion molecules and was implicated in cell recognition and axonal guidance (Rauch *et al.*, 1991). In *Drosophila*, the analogous HRP carbohydrate epitope was found in neural recognition molecules as well as in receptor protein tyrosine phosphatases that are expressed in the developing nervous system (Desai *et al.*, 1994). It was demonstrated recently that loss-of-function mutations in *Drosophila* RPTPs result in erroneous pathfinding of certain motor axons (Desai *et al.*, 1996; Krueger *et al.*, 1996).

In our attempts to identify specific ligands of RPTP β , we used soluble, recombinant CAH or FNIII domains of this receptor phosphatase as specific reagents for the identification of cellular proteins that bind to RPTP β . We have demonstrated that the FNIII repeat binds specifically to glial cells while the CAH domain of RPTP β binds to neurons or cells of neuronal origin (Peles *et al.*, 1995). We identified contactin, a glycosylphosphatidylinositol (GPI)-anchored recognition receptor, as the neuronal-specific cell surface protein that interacts with the CAH domain of RPTP β (Peles *et al.*, 1995). Contactin belongs to a group of neuronal recognition molecules (including TAG-1 and BIG-1) that contain both immunoglobulin (Ig)-like domains and FNIII repeats and appear to mediate interactions between neurons and their local environment during development (Dodd *et al.*, 1988; Rathjen and Jessel, 1991; Yoshihara *et al.*, 1994). Indeed, we have demonstrated that the CAH domain of RPTP β mediates cell adhesion and neurite outgrowth of primary tectal neurons and neuroblastoma cells (Peles *et al.*, 1995). These responses were blocked by antibodies against contactin, demonstrating that contactin is the neuronal receptor for the CAH domain. These experiments suggested that the interaction between RPTP β and contactin can lead to bidirectional signals between neurons and glial cells in the developing nervous system.

Signaling by cell recognition molecules has been shown

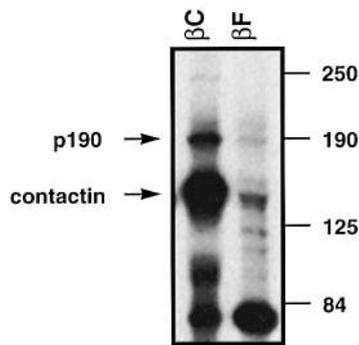


Fig. 1. Association between contactin and p190 in membranes from GH3 cells. GH3 rat neuroendocrine cells were metabolically labeled with [35 S]methionine and membrane proteins were subjected to precipitation with Ig fusion proteins containing the CAH domain (β C) or the FNIII repeat (β F) of RPTP β as indicated. Washed complexes were resolved on a 7.5% SDS gel. An autoradiogram of the fixed and dried gel is shown. The location of molecular mass markers is given in kDa and the location of contactin and p190 is indicated.

to utilize a variety of intracellular signaling systems including GTP-binding proteins, calcium influx and protein kinases (reviewed in Doherty and Walsh, 1994). It was proposed that members of the Src family of protein tyrosine kinases are involved in the transmission of signals generated at the cell surface by cell recognition molecules such as L1 and N-CAM (Beggs *et al.*, 1993; Ignelzi *et al.*, 1994), MAG (Umemori *et al.*, 1994) and contactin (Olive *et al.*, 1995; Zisch *et al.*, 1995). Since contactin is a GPI-anchored protein lacking a cytoplasmic domain, it may mediate its biological responses by interaction with other membrane receptors that are able to recruit and activate intracellular signaling molecules.

We previously have identified a 190 kDa protein that co-precipitates with contactin bound to the CAH domain of RPTP β . We have proposed that this protein may participate in contactin-mediated signaling. Here we describe the cloning and characterization of p190 and its mode of interaction with contactin.

Results

We have demonstrated previously that the CAH domain of RPTP β binds specifically to contactin which is expressed on the surface of neuronal cells, leading to neurite outgrowth and differentiation (Peles *et al.*, 1995). When a soluble CAH-Fc fusion protein (β C-Fc) was used for the purification of contactin, we found an additional protein of an apparent M_r of 180–190 kDa (p190) to be associated with the CAH–contactin complex (Peles *et al.*, 1995 and Figure 1). We reasoned that this p190 protein may play a role in cellular signaling mediated by contactin, and decided to purify it using affinity chromatography with β C-Fc. Membrane lysates from GH3 cells were applied to a β C-Fc column and bound proteins were separated by SDS–PAGE. The protein band corresponding to p190 was excised and subjected to trypsin digestion. The amino acid sequences of two tryptic peptides were determined using a gas-phase microsequencer (Figure 2A). The sequence of one of the peptides (QNLPQILEES) matched the sequence of an expressed sequence tag (EST) fragment from the *BRCA1* region of human chromosome 17q21 (Friedman *et al.*, 1994) in GeneBank. This DNA

fragment was isolated by PCR and used as a probe for cloning the human and rat cDNAs of p190. As shown in Figure 2A, rat and human p190 have open reading frames of 1384 and 1381 amino acids respectively. Alignment of both amino acid sequences shows that human and rat p190 exhibit 93% sequence identity. The p190 protein has hallmarks of a type I transmembrane receptor. The first methionine is followed by a stretch of 19–20 amino acids rich in hydrophobic residues and probably functions as a signal sequence. The extracellular domains of rat and human p190 contain 1281 and 1282 amino acids respectively, with 16 potential *N*-linked glycosylation sites followed by a second hydrophobic stretch that is a typical transmembrane domain. Both human and rat p190 have short cytoplasmic domains composed of 74 or 78 amino acids respectively.

The extracellular domain of p190 is a mosaic of distinct sequence motifs that were shown to be involved in protein–protein interactions (Figure 2B). The amino-terminus of p190 contains a stretch of 109 amino acids with 31–33% identity to the terminal domains of coagulation factors V and VIII (Wood *et al.*, 1984; Jenny *et al.*, 1987), 26% identity with the neuronal adhesion molecule neuropilin (also known as A5 antigen; Takagi *et al.*, 1991) and 20% identity to a region in discoidin I, a lectin from the slime mold *Dictyostelium discoideum* (Figure 2C). The extracellular domain of p190 also contains four repeats of ~140 amino acid residues each with 29–32% sequence identity to the extracellular domains of the neuronal cell surface proteins neurexins. Similar motifs were also found in laminin A, agrin, slit and perlecan (Ushkaryov *et al.*, 1992). This region in laminin A is referred to as the G domain, and is thought to mediate cell adhesion. The first three neurexin repeats of p190 share 29–32% amino acid identity with sequences in rat neurexin III- α and neurexin II- α , whereas the fourth repeat is most similar to a sequence in agrin (34% identity). In addition, the extracellular domain of p190 contains two epidermal growth factor (EGF)-like repeats homologous to those found in the *Drosophila* neurogenic proteins Notch and Slit (39–46% identity) (Wharton *et al.*, 1985; Vassin *et al.*, 1987). Moreover, the second neurexin repeat of p190 and the first EGF-like repeat flank a stretch of 158 amino acids homologous to a region in fibrinogen β/γ . Finally, the extracellular domain of both rat and human p190 contain an identical sequence of 47 amino acids composed of eight repeats of the sequence Pro–Gly–Tyr. A similar repeat was found in a molluscan adhesive protein (Waite *et al.*, 1989) and in a putative chicken prion protein (Gabriel *et al.*, 1992).

The cytoplasmic domains of human and rat p190 contain 78 and 74 amino acids, respectively. These include a stretch of 38–42 amino acids rich in proline residues (38%), the majority of which consist of proline alternating with alanine, glycine or threonine residues.

Expression of p190 mRNA

Northern blot analysis of mRNA isolated from human tissues shows that p190 was expressed predominantly in the brain as a transcript of 6.2 kb (Figure 3A, left panel). Weak expression of p190 was detected in the ovary as well as in the pancreas, colon, lung, heart, intestine and testis. Similar results were obtained when a probe from

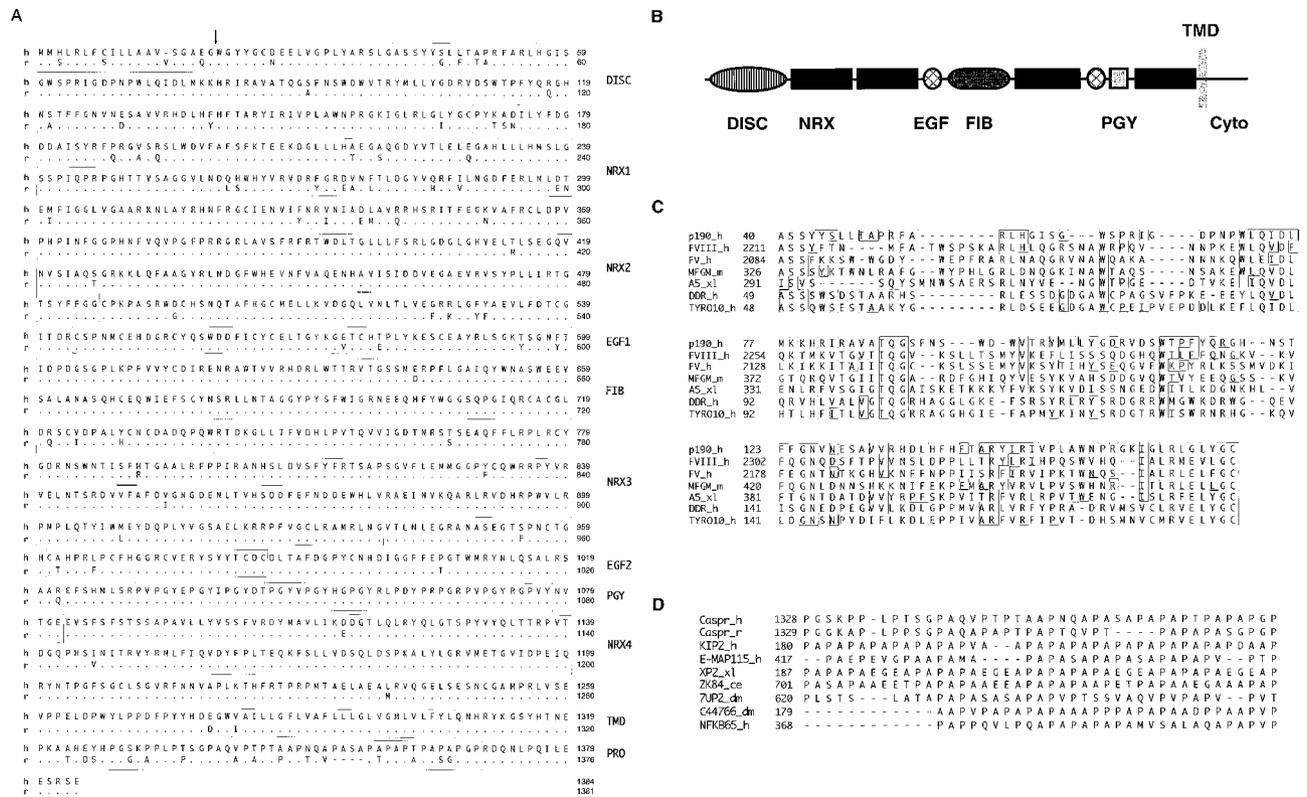


Fig. 2. Deduced amino acid sequences of human and rat p190. (A) The amino acid sequence of human p190 (h) is shown in a single-letter amino acid code and numbered on the right. Amino acids that are different in the sequence of rat p190 (r) are indicated. Peptide sequences identified by protein microsequencing are LFIQVDYFPLTEQ and QNLPQILEES at positions 1156 and 1369 respectively. The end of the hydrophobic signal sequence is marked by an arrow. Different domains in the protein are framed and labeled as follows: DISC, discoidin/factor V homology region; NRX, neurexin-like domains; FIB, fibrinogen β/γ -related sequence; EGF, epidermal growth factor-like repeat; PGY, glue domain containing PGY repeats; TMD, transmembrane domain; PRO, proline-rich region. (B) Schematic representation of the different structural motifs. Cyto, cytoplasmic tail. (C) Alignment of the N-terminal discoidin domain of p190 with homologous sequences in other proteins. Amino acids 40–173 of human p190 are aligned with the C2 domain of coagulation factor V (FV, PIR A56172; Jenny *et al.*, 1992), factor VIII (FVIII, PIR A00525; Wood *et al.*, 1984), the milk fat globule membrane protein (MFG-F8; PIR A35479; Stubbs, 1990), the b1 region of *Xenopus* neurophilin, also known as the neuronal A5 antigen (A5; PIR JH0466; Takagi *et al.*, 1991) and with two receptors tyrosine kinases (DDR; GBL 11315; Johnson *et al.*, 1993 and Tyro10; PIR S42621; Karn *et al.*, 1993). (D) Alignment of the cytoplasmic region of p190 with other proteins containing Pro-Ala-Thr-rich ('PAPA') sequences: KIP2 is a human cyclin-dependent kinase inhibitor (GB U22398; Matsuoka *et al.*, 1995); E-MAP115, a human microtubule-associated protein (GB X73882; Masson and Kreis, 1993); XP2, a secretory protein in *Xenopus* skin (SW P17437; Hauser *et al.*, 1992); ZK84, a putative protein in *Caenorhabditis elegans* (GB U23181; Wilson *et al.*, 1994); 7UP, a nuclear hormone receptor important for eye development in *Drosophila* (SW P16376; Mlodzik *et al.*, 1990); fc177, an eggshell protein in *Drosophila* (PIR C44766, Waring *et al.*, 1990); and NFKB65, the p65 subunit of the transcription factor NF- κ B (GB L19067; Lyle *et al.*, 1994).

rat p190 was used in Northern blot analysis performed with mRNA isolated from different rat tissues (data not shown). A high level of expression of p190 was detected in different regions of the adult human nervous system. The Northern blot presented in Figure 3B shows that p190 is highly expressed in the cortex, cerebellum and in the thalamus, while weaker expression is detected in the spinal cord and in the corpus callosum. These analyses demonstrated that p190 is expressed predominantly in the nervous system.

Expression of p190 protein

Polyclonal rabbit antibodies were raised against a GST fusion protein containing the cytoplasmic domain of p190. This antiserum specifically stained permeabilized human IMR-32 neuroblastoma and rat GH3 neuroendocrine cells, two cell lines that express p190 (Figure 4A). When used for immunoprecipitation and immunoblot analysis, the anti-p190 antiserum recognized a protein that migrated in SDS gels with an apparent mol. wt of 190 kDa (Figure 4B). Similar results were obtained

when immunoprecipitation and immunoblotting analysis was performed with lysates of COS7 cells that were transfected with an expression vector that directs the synthesis of p190 (Figure 4B). The p190 protein was not detected in lysates from mock-transfected or untransfected COS7 cells.

We next used immunohistochemistry to compare the localization of p190 and contactin in the rat retina. Staining of neonatal rat retina with anti-p190 antibodies demonstrated specific staining in the ganglion cell fiber layer and the inner plexiform layers (Figure 4C). Histochemical analysis with antibodies against contactin revealed similar staining, with highest expression in the nerve fiber layer containing the axons that project from the ganglion cells into the optic nerve (Figure 4C). These experiments demonstrate that p190 and contactin were co-localized on neurons in fiber-rich areas of the retina and are consistent with previous studies demonstrating expression of contactin in several distinct layers of the retina, including the ganglion cell fibers, and at the inner plexiform layer (D'Alessandri *et al.*, 1995). Increased

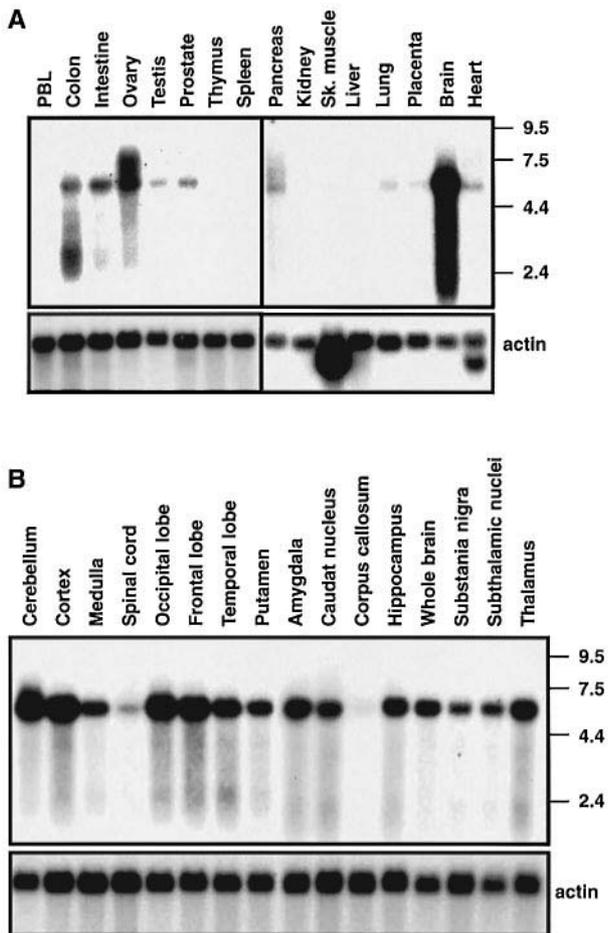


Fig. 3. Expression of p190 mRNA in different tissues. Northern blot analysis of mRNA from different human tissues (A) and from eight different human brain sections (B). The upper panel in each figure depicts Northern blots that were probed with a p190-specific probe. The lower panel shows control autoradiograms of the same blots after hybridization with a human β -actin probe. The sizes of molecular weight markers are shown in kb on the right.

expression of p190 was also detected in membrane preparations from rat brains from E18 to post-natal day eight, a period of extensive axonal outgrowth and synaptogenesis (data not shown). A similar temporal expression of contactin was detected in this tissue in the same period (Gennarini *et al.*, 1989).

Lateral interaction in the plasma membrane between p190 and contactin

We next examined the interaction between contactin, RPTP β and p190 using soluble and membrane-associated variants of these proteins. The CAH domain of RPTP β binds to contactin-expressing cells, and a soluble form of contactin can bind to transmembrane forms of RPTP β (Figure 5A and Peles *et al.*, 1995). However, neither soluble contactin nor the CAH domain of RPTP β could bind to COS7 cells over-expressing p190 protein on their surface (Figure 5A). This result raised the possibility that the interaction between contactin and p190 requires that both proteins be present on the surface of the same cell (*cis* interactions). To examine this possibility, COS7 cells were transfected with expression vectors that direct the synthesis of either p190 alone or together with contactin. Lysates of the transfected cells were

subjected to precipitation analysis with the CAH domain of RPTP β (β C-Fc). The experiment presented in Figure 5B shows that the CAH domain of RPTP β only precipitated p190 from cells co-expressing contactin. It thus appears that the CAH domain of RPTP β may form a ternary complex with contactin and p190 proteins. Similar results were obtained using an expression vector that directs the synthesis of p190 tagged with hemagglutinin epitope (HA-tag). The experiment presented in Figure 5C demonstrates that tagged p190 was only precipitated by the CAH domain from lysates of cells co-expressing both contactin and p190 molecules. Moreover, soluble contactin molecules did not associate with p190 when RPTP β and p190 were co-expressed in the same cells (Figure 5C). On the basis of these experiments, we propose that the CAH domain of RPTP β does not bind directly to p190 and that contactin and p190 are complexed by means of lateral interactions (*cis*) in the membrane. We therefore refer to p190 as Contactin-associated protein (Caspr).

Complex formation between Caspr and contactin

We next examined the role of RPTP β in formation of the complex between Caspr and contactin. Lysates from IMR-32 cells were subjected to immunoprecipitation with antibodies against Caspr followed by immunoblotting with antibodies against contactin. These experiments demonstrated that contactin and Caspr were constitutively associated on the surface of IMR-32 cells (Figure 6A). We observed that anti-contactin antibodies or β C-Fc were able to precipitate similar amounts of contactin, while not more than 10–20% of contactin molecules were detected in immunoprecipitates of anti-Caspr antibodies. Since the efficiency of the immunoprecipitation reaction was very high (essentially all Caspr molecules were precipitated), we estimated that virtually all Caspr molecules were associated with contactin in this cell line. This analysis indicated that contactin molecules were in excess and ~80% of the contactin molecules were not associated with Caspr.

We also investigated the existence of a contactin–Caspr complex *in vivo* in rat brain tissue. Lysates of P7 rat brain membranes were subject to precipitation with β C-Fc followed by immunoblotting with antibodies specific to either contactin or Caspr. The experiment presented in Figure 6B demonstrated that contactin and Caspr formed a stable complex in lysates from rat brain. Moreover, immunoprecipitates of contactin that were subjected to immunoblot analysis with anti-Caspr antibodies also revealed the presence of Caspr protein. Similarly to the IMR-32 cells, rat brain lysates have an excess of contactin over Caspr, and the complex formation between these two proteins is limited by the concentration of Caspr.

Taken together, these experiments demonstrate that contactin and Caspr are constitutively complexed in neuronal cell lines and tissues and that complex formation between these two proteins does not require RPTP β .

Interaction between Caspr and SH3 domains of signaling molecules

Contactin is a GPI-linked cell surface molecule, and therefore likely to mediate signals across the plasma membrane by lateral interactions with a transmembrane co-receptor containing a cytoplasmic domain that can

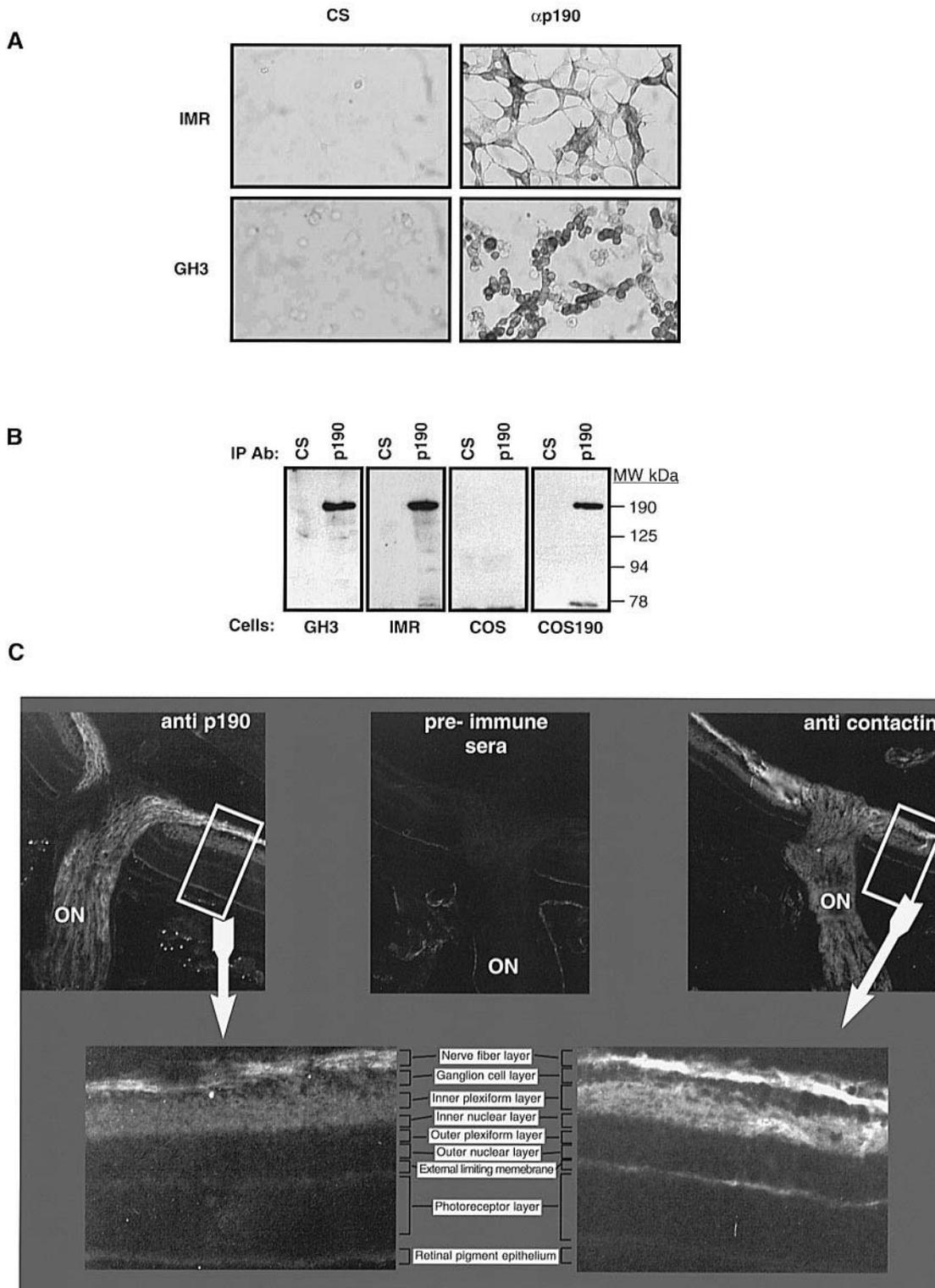


Fig. 4. Expression of p190 protein. **(A)** Staining of IMR-32 neuroblastoma and GH3 neuroendocrine cell lines with antibodies against p190 (α p190) or with pre-immune serum as control (CS). Antibodies were detected using biotinylated secondary antibodies and alkaline phosphatase-conjugated streptavidin. **(B)** Immunoblot analysis of p190 from IMR-32 and GH3 cell lines and from COS7 cells that were transfected with rat p190 expression vector or untransfected COS7 as control. Antibodies against p190 or control sera (CS) were used for immunoprecipitation followed by Western blotting with antibodies against p190. Blots were detected with peroxidase-linked protein A and chemiluminescence reagent. **(C)** Expression of p190 and contactin in rat retina. Immunohistochemistry with antibodies against p190 (left panels) or against contactin/F3 (right panels) was performed as described in Materials and methods. Staining with pre-immune serum (middle panel) showed non-specific staining on the external limiting membrane. The different layers are indicated. ON, optic nerve.

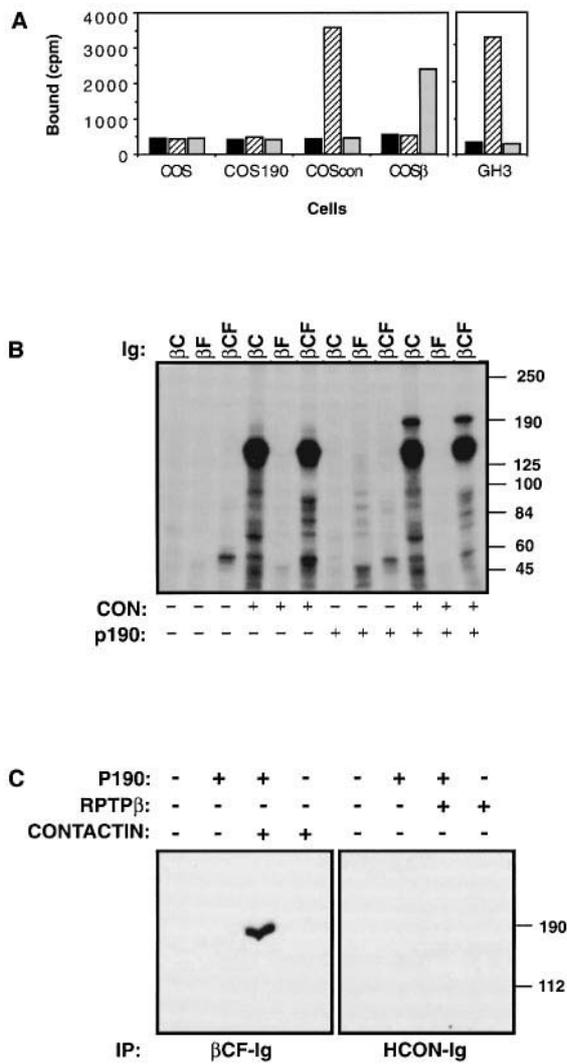


Fig. 5. Association between p190 and contactin expressed on the surface of the same cells. (A) Binding of soluble contactin (HCON-Fc) and CAH domain of RPTPβ (βC-Fc) to cells expressing Caspr. COS7 cells were transfected with the short form of RPTPβ (COSβ), human contactin (COScon), rat p190 (COS190), or with βGAL as control (COS). Control medium (filled rectangles) or medium containing βC-Fc fusion protein (striped rectangles) or HCON-Fc fusion protein (shaded rectangles), both at 0.5 μg/ml was used. Bound fusion proteins were detected by using [¹²⁵I]protein A. The same Ig fusion proteins were used for binding experiments on GH3 cells (right panel). (B) and (C) Reconstitution of the interactions between p190 and contactin in transfected cells. COS7 cells were transfected with the indicated expression vectors and subsequently metabolically labeled with [³⁵S]methionine. Solubilized membranes were subjected to precipitation with an Ig fusion protein containing either the FNIII domain (βF), the CAH domain (βC) or both domains (βCF) of RPTPβ as indicated. Washed complexes were separated on a 7.5% SDS gel. An autoradiogram of the fixed and dried gel is shown in (B). The locations of molecular mass markers are indicated in kDa. (C) COS7 cells were transfected as described above with an HA-tagged version of p190 alone or together with contactin or RPTPβ as indicated (C; -/+). Solubilized membranes were precipitated with βC-Fc followed by immune blotting with antibody against the HA-tag. The blots were detected with peroxidase-linked anti-mouse antibodies and chemiluminescence reagent.

recruit or activate intracellular signaling molecules. Caspr is a good candidate for serving such a function since it contains a cytoplasmic domain and is able to associate with contactin. The cytoplasmic domain of Caspr contains

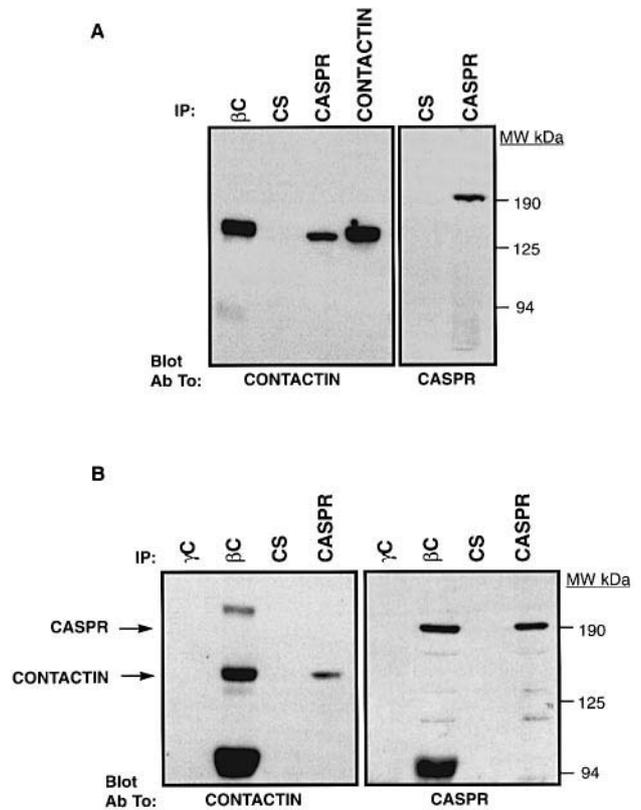


Fig. 6. Detection of the Caspr–contactin complex in cell lines and in rat brain tissue. (A) Membrane proteins from IMR-32 neuroblastoma cells were precipitated with Ig fusion protein containing the CAH domain of RPTPβ (βC), with control pre-immune serum (CS), with anti-Caspr antibodies (CASPR) or antibodies against contactin (CONTACTIN) as indicated. Complexes were resolved by SDS–PAGE and blotted with antibodies against contactin (left panel) or against Caspr (right panel). (B) Caspr and contactin form a complex in rat brain tissue. Solubilized membranes were prepared from P7 rat brains as described in Materials and methods. Equal amounts of proteins were subjected to precipitation with the above-mentioned reagents as indicated. In addition, an Ig fusion protein containing the CAH domain of RPTPγ was used as a control (γC). Immunoblotting was performed with antibodies against contactin or Caspr as indicated. The location of contactin (p140) and Caspr (p190 on the gel) are indicated by arrows.

a proline-rich sequence with at least one canonical SH3 domain-binding site. A reasonable hypothesis, therefore, is that the cytoplasmic domain of Caspr can serve as a binding site for SH3 domains of signaling molecules which will transmit the signal initiated by RPTPβ binding to the contactin–Caspr complex.

We tested this hypothesis by examining the ability of a variety of GST–SH3 domains of signaling molecules to interact with Caspr *in vitro*. Figure 7A shows that four out of seven GST–SH3 domains tested were able to bind selectively to the Caspr protein, including the SH3 domains of Src, Fyn, p85 and PLCγ. Association was not detected between Caspr and the SH3 domains of Csk, Grb2 and Gap. Moreover, Caspr did not bind to a mutant Src SH3 domain in which a conserved tryptophan at position 118 was replaced by an alanine residue (W118A). We next examined whether c-Src could associate with Caspr in transiently transfected cells. COS7 cells were transfected with an expression vector that directs the synthesis of c-Src together with an expression vector for Caspr-HA.

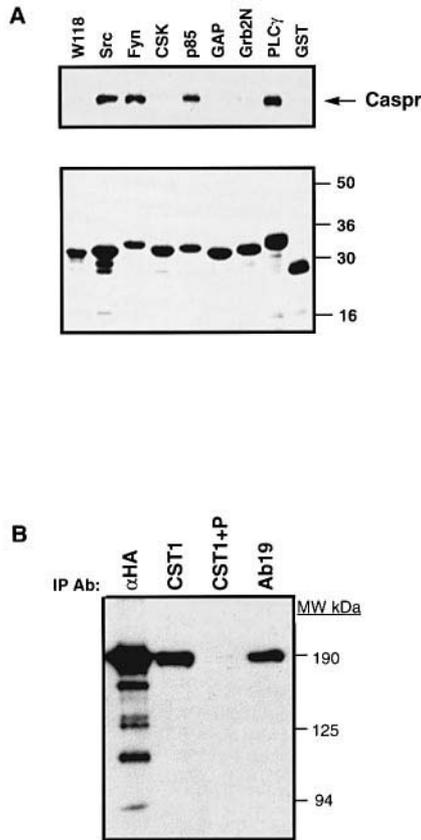


Fig. 7. Interaction of Caspr with SH3 domains of signaling proteins. **(A)** Precipitation of Caspr using GST-SH3 fusion proteins. Lysates of COS7 cells expressing an HA-tagged version of rat Caspr were incubated with the indicated GST fusion proteins coupled to glutathione-agarose. Washed complexes were separated on a 7.5% SDS gel and blotted with antibody against HA-tag (upper panel). The following GST fusions were used: GST alone (GST), Csk, Fyn, a point mutant Src in which a tryptophan residue at position 118 was replaced with an alanine residue (W118A), Src, p85 subunit of PI3K, GAP, PLC γ and the N-terminal SH3 domain of Grb2 (Grb2N). The lower panel shows a Coomassie-stained gel of the recombinant proteins used for precipitation. **(B)** Co-precipitation of Caspr with Src kinase. COS7 cells were transfected with an HA-tagged version of rat Caspr along with *src* expression vector. Cells were lysed in a buffer containing 1% Triton X-100 and subjected to immunoprecipitation with either polyclonal antibodies against Src (Ab19 and CST1), pre-immune antiserum (CS) or antibody against the HA-tag. As an additional control for specificity, the peptide that was used for generation of CST1 antibodies was included in the immunoprecipitation (CST1+pep) mixture. Immunocomplexes were separated on a 7.5% SDS gel and immunoblotted with antibodies against HA-tag. The blots were then reacted with peroxidase-linked anti-mouse antibodies and detected by chemiluminescence reagent. For immunoprecipitation with the anti-HA antibody, only 10% of the lysate was used.

Lysates of the transfected cells were subjected to immunoprecipitation with antibodies against c-Src, followed by immunoblotting with anti-HA antibodies. The experiment presented in Figure 7B shows that Caspr can be co-precipitated with several different c-Src-specific antibodies. Moreover, the co-precipitation was completely blocked in the presence of the synthetic peptide that was used for the generation of anti-Src antibodies.

We also examined the association between endogenous c-Src and Caspr in IMR-32 or GH3 cells using a similar immunoprecipitation/immunoblotting strategy utilizing

anti-Src and anti-Caspr antibodies. We demonstrated that the anti-Src and anti-Caspr antibodies recognized their cognate antigens in these cells. However, association between endogenous c-Src and Caspr could not be detected. Similar negative results were obtained when polyclonal rabbit antibodies that recognize several known members of the Src family kinases were used in the immunoprecipitation experiment (data not shown).

These experiments raise the possibility that the cytoplasmic domain of Caspr may serve as a target for SH3 domains of signaling molecules. However, the physiologically relevant signaling molecules that bind to Caspr probably remain to be identified.

Discussion

Here we describe the identification and cloning of Caspr, a novel cell surface protein that interacts with the GPI-linked cell recognition molecule contactin by means of lateral association in the plasma membrane. We demonstrate that the CAH domain of RPTP β binds to contactin but not to Caspr. However, contactin can bind simultaneously to both Caspr and the CAH domain of RPTP β , leading to formation of a complex composed of these three proteins. Caspr is a transmembrane protein with a variety of subdomains found in proteins that have been implicated in synaptogenesis, axonal guidance and target recognition.

The overall architecture of the extracellular domain of Caspr is similar to the structure of the extracellular domains of neurexins, a family of highly polymorphic cell surface molecules (Ushkaryov *et al.*, 1992; Ullrich *et al.*, 1995). Both neurexins and Caspr contain EGF-like repeats and sequences with homology to the extracellular matrix proteins laminin A, agrin, slit and perlecan (Sasaki *et al.*, 1988; Rothberg *et al.*, 1990; Noonan *et al.*, 1991; Rupp *et al.*, 1991; Ushkaryov *et al.*, 1992). The region in laminin A (the G domain) that is homologous to Caspr is thought to mediate the binding of laminin to cell surface integrin and heparin (Timpl, 1989; Gehlsen *et al.*, 1992). The N-terminal portion of Caspr contains a region homologous to the C2 domain of coagulation factors V and VIII (Wood *et al.*, 1984; Jenny *et al.*, 1987). It has been proposed that this domain mediates the attachment of these factors to phospholipids on the cell surface of platelets where they interact with other factors of the coagulation cascade (Kemball-Coak *et al.*, 1988). Finally, the extracellular domain of Caspr contains two EGF-like repeats that are most similar to those found in the neurogenic genes notch and delta (Wharton *et al.*, 1985; Vassin *et al.*, 1987). Interestingly, EGF-like repeats were also found in coagulation factors, in agrin and in neurexins; all these proteins share additional regions of homology with Caspr.

We have analyzed the mode of interaction between Caspr and contactin by expressing these two proteins alone or together in transfected cells. These experiments demonstrated that contactin and Caspr form a complex only when the two proteins are expressed on the surface of the same cell. Moreover, the association between these two proteins appears to be constitutive and independent of the binding of the CAH domain to contactin molecules. We also found that in cultured cell lines, as well as in

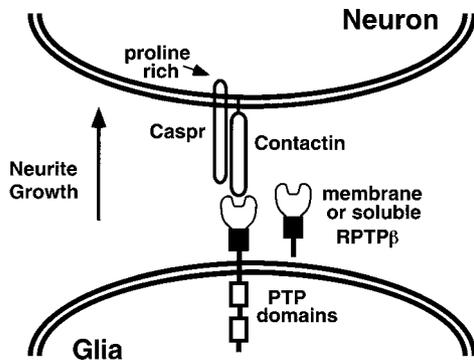


Fig. 8. A proposed model of the interactions between RPTP β , contactin and Caspr. Soluble or membrane forms of RPTP β expressed on the cell surface of glial cells bind to the GPI-anchored recognition receptor contactin present on the cell surface of neuronal cells. Contactin forms a stable complex with the transmembrane receptor Caspr on certain neurons. The cytoplasmic domain of Caspr is rich in proline residues. These regions may serve as binding sites for SH3 domains of signaling proteins.

rat brain tissue, complex formation was limited by the concentration of Caspr and that the majority of contactin molecules (~80%) were not bound to Caspr. The ability of Caspr and contactin to interact when both are expressed on the surface of the same cell may suggest that these two proteins function as two subunits of a signaling receptor (Figure 8).

Caspr is a good candidate for serving as a signaling subunit of contactin; the cytoplasmic domain of Caspr may allow recruitment or activation of signaling molecules leading to signal transmission from contactin into the cell interior. The cytoplasmic domain of Caspr contains proline-rich sequences which serve as binding sites for SH3 domains of signaling molecules. Our studies demonstrate a selective association between the cytoplasmic domain of Caspr and the SH3 domains of Src, Fyn, p85 and PLC γ . We also demonstrated association between c-Src and Caspr upon transient overexpression of these two proteins in transfected COS7 cells. However, the same co-immunoprecipitation strategy failed to reveal association between endogenous Caspr and members of the Src family in IMR-32 and GH3 cells. The identity of the physiologically relevant SH3 domain-containing signaling molecules that bind to Caspr remains to be determined. It is also possible that sequences other than the proline-rich regions of the cytoplasmic domain of Caspr may also be involved in recruiting signaling proteins through a different mode of protein-protein interaction.

It is possible that Caspr may bind to other members of the contactin family of cell recognition receptors and thus serve as a signaling subunit of additional GPI-linked recognition receptors. We have found that only a minority of contactin molecules are bound to Caspr in cultured cells and in neuronal tissue. Previous studies have demonstrated association between contactin and Ng-CAM and Nr-CAM (Brümmendorf *et al.*, 1993; Morales *et al.*, 1993). These two proteins are also transmembrane receptors and potentially could couple intracellular signaling molecules to contactin by means of lateral interaction in the plasma membrane. Accordingly, Ng-CAM was shown previously to be linked to intracellular second messenger systems

(reviewed in Doherty and Walsh, 1994). It is not clear yet whether these interactions can affect the interaction between contactin and Caspr and vice versa.

Surface molecules play a key role in axonal pathfinding, target recognition and in the generation and maintenance of synapses (Sonderregger and Rathjen, 1991; Garrity and Zipursky, 1995). The rearrangement of cell surface composition is therefore one of the means of controlling the specificity and diversity of cell-cell interactions. Such rearrangement will enable the growing axon to respond to changes in the environment. An alternative mechanism involved regulated changes in the ability of a single recognition molecule to interact with a given ligand. Although it seems that the presence of Caspr does not affect the binding of the CAH domain of RPTP β to contactin, it may regulate the interaction of contactin with other ligands such as the extracellular matrix proteins tenascin and restrictin and the recognition molecules Ng-CAM and Nr-CAM.

The assembly of functional cell surface receptors is a common theme in transmembrane signaling. It is now well established that oligomerization is essential for the activation of both growth factor and lymphokine receptors. Heterodimerization of cell surface components could also provide a simple mechanism for generation of signal diversity (Lemmon and Schlessinger, 1994). Hence, the specific association between contactin and several transmembrane receptors may increase the repertoire of signals generated by this neuronal recognition molecule. The complex interactions between RPTP β , contactin and Caspr delineate one mechanism by which neuronal and glial cells communicate in the developing nervous system.

Materials and methods

Protein purification and sequencing

Solubilized membrane lysate was prepared from 3×10^9 GH3 cells and loaded on a column of β CF-Fc bound to Sepharose protein A (Pharmacia) as described previously (Peles *et al.*, 1995). Bound proteins were separated on 6.5% SDS gel, blotted to ProBlot membrane (Applied Biosystems, Inc.) and stained with Coomassie R-250. To obtain the internal peptide sequence, the blotted 190 kDa band was digested for 16 h with 1 μ g of modified trypsin (Promega) in 50 μ l of 0.1 M Tris pH 8.0, 10% acetonitrile, 1% octylglucoside. Digestion was stopped by the addition of 2 μ l of neat trifluoroacetic acid (TFA) and peptides were separated on a 1 mm \times 200 mm Reliasil C-18 reverse phase column on a Michrom UMA HPLC. Purified peptides were sequenced as described (Peles *et al.*, 1995).

Cloning of rat and human Caspar cDNA

The sequence of one tryptic peptide obtained from the purified protein (QNLQPQILEES) was found in a 900 bp EST fragment (B102/LF98) from the *BRAC1* region on chromosome 17q21 in GeneBank (Friedman *et al.*, 1994). Primers corresponding to this region (5' primer: TCG CAG GCT ATG AGC CTG GCT ACA TCC; 3' primer: GTG GGT AGG GGA GGT TTG CTG CCA GG) were used for RT-PCR to clone this DNA fragment from rat GH3 cells. A 600 bp DNA fragment derived from this region was used further as a probe for screening a ZAPEX-GH3 cDNA library. This cDNA library was constructed in ZAP-Express phage (Stratagene, San Diego, CA), using oligo(dT) priming. Hybridization and other cloning techniques were performed according to standard procedures. Clone ZX5 had a 2.5 kb insert containing a sequence that matched the sequence of the second tryptic peptide from p190. A second cDNA library was constructed from GH3 mRNA by priming with a specific oligonucleotide GGA GGT CTC CTT TAG according to the sequence that was found in the 5' end of clone ZX5. This cDNA was cloned into ZAP-Express (Stratagene, San Diego, CA) to generate the ZB-GH3 library. This library was used for isolating multiple clones that overlapped with ZX5 and contained the 5' end of

the gene. To clone human p190, a cDNA library was constructed from mRNA that was isolated from IMR-32 neuroblastoma cells in ZAP-Express (ZX-IMR). Probes were generated by PCR from the 5' ends of rat clone ZB181 and from IMR-32 cDNA according to the B102 sequence as described above for the rat gene. Several clones had a 5 kb insert that contained the full-length gene. DNA sequence determination was carried out using the dideoxy chain termination method with Sequenase 2.0 (United States Biochemical Corporation, Cleveland, OH). Sequencing was performed on both strands by priming with synthetic oligonucleotides.

Expression constructs

An *EcoRI*–*XhoI* fragment containing the 5' end of rat *CASPR* (from clone ZB161) was ligated with an *XhoI*–*EcoRI* fragment containing the 3' end of the gene (from clone ZB181) and cloned into pCMP1 (Peles *et al.*, 1995) to generate pCM190R. An HA-tagged version of the gene was constructed by replacing an *EcoRI*–*AccI* fragment with a PCR-generated fragment containing the HA-tag sequence. This resulted in the addition of the HA sequence to the 3' end of the coding region of rat *CASPR* to generate pCM190HA. Construction of contactin expression vectors was described previously (Peles *et al.*, 1995). The plasmid pSGT-cSRC containing the human *src* gene and plasmids used for generation of the GST–SH3 fusion proteins were described previously (Erplel *et al.*, 1995). To generate a GST fusion protein containing the cytoplasmic tail of rat Caspr, the corresponding region (amino acids 1308–1380) was amplified by PCR and cloned into pGEX-4T (Pharmacia). The sequence of the final construct was verified by DNA sequencing.

Northern blot analysis

A DNA fragment (position 3600–4232 of human *CASPR*) was generated by RT–PCR from IMR-32 mRNA. This fragment was labeled by random priming ('prime it'; Stratagene, San Diego, CA), purified using a PCR-clean column (Qiagen) and used as a probe. Hybridization to multiple tissue Northern blots (MTN Blots, Clontech) was carried out for 16 h in a buffer containing 5× SSC, 5× Denhardt's solution, 50% formamide, 0.2% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C. The blots were washed twice at 60°C in a buffer containing 0.5× SSC, 0.1% SDS and once with 0.1× SSC, 0.2% SDS. Signals were detected by autoradiography. The same membranes were reprobed with a 2 kb human β-actin cDNA as a control.

Ig fusion proteins and immunohistochemistry

Production of different Ig fusion proteins and cell binding experiments were performed as described previously (Peles *et al.*, 1995). Immunohistochemical staining of tissue sections with antibodies was done essentially as described (Milev *et al.*, 1994).

Generation of antibodies

Polyclonal antibodies against p190/Caspr were generated by immunizing rabbits with a GST fusion protein composed of the entire cytoplasmic domain of rat p190/Caspr (GST–190CT). Antibodies against GST were removed by passing the antiserum on a column of Sepharose–GST. Affinity purification was then performed by loading the unbound material on a column of GST–190CT Sepharose. Bound antibodies were eluted with 100 mM sodium citrate pH 2.8 and 1.5 M MgCl₂. Eluted material was precipitated with ammonium sulfate, resuspended in distilled water and dialyzed extensively against phosphate-buffered saline (PBS). Antibody 87AP was generated against a synthetic peptide of eight amino acids from the C-terminus of rat Caspr. Affinity purification on a Sepharose–peptide column was done essentially as described above for Ab60. Anti-F3 antibodies were obtained from G.Rougon (Favre-Sarrailh *et al.*, 1992). Polyclonal antibodies CST1 that recognize Src, Fyn and Yes were previously described (Erplel *et al.*, 1995). Ab18 and Ab19 against Src were purchased from Santa Cruz Antibodies (Santa Cruz, CA). Monoclonal antibody against HA-tag was purchased from Boehringer Mannheim (Indianapolis, IN). Mouse polyclonal antibodies against contactin were generated by immunization of mice with purified human contactin–Ig fusion protein according to Yoshihara *et al.* (1994).

Immunoprecipitation and immunoblot analysis

To detect the association between contactin and Caspr, the cells were grown to subconfluency and were metabolically labeled with 100 mCi/ml [³⁵S]methionine and cysteine mix (NEN, Boston, MA) for 4 h at 37°C. Membranes were prepared from the cells and solubilized further in SML buffer [2% sodium monolaurate, 2 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride (PMSF) in PBS]. βC-Fc bound to protein A–Sepharose beads was added to a 10-fold diluted supernatant and incubated for 2 h

at 4°C. The beads were washed twice with 0.15% sodium monolaurate in PBS and once in PBS before the addition of SDS sample buffer. The precipitated proteins were separated on 7.5% gel and subjected to autoradiography.

For the preparation of rat brain membranes, five rat brains (P7) were pooled and homogenized in a glass homogenizer in a buffer containing 20 mM HEPES pH 7.4, 0.32 M sucrose, 1 mM EGTA, 1.5 mM MgSO₄, 10 µg/ml aprotinin and leupeptin and 1 mM PMSF. Nuclei and heavy cell debris were removed by low speed centrifugation (3000 g for 10 min at 4°C), and the supernatant was subjected to high speed centrifugation at 40 000 g for 60 min. The membrane pellet was resuspended in SML buffer. After 1 h incubation on ice, the detergent-insoluble material was removed by centrifugation. The sample was diluted 4- to 10-fold with PBS containing 2 mM MgCl₂ and subjected to precipitation with antibodies or Ig fusion proteins. Immunoprecipitation and immunoblotting were performed as described previously (Peles *et al.*, 1991).

Accession numbers

The sequences described in this paper have been submitted to the DDBJ/EMBL/GenBank database under accession numbers U487223 and U487224.

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