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Interaction of Rat Lin-10 with Brain-Enriched F-Actin-Binding Protein, Neurabin-II/Spinophilin

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We have recently isolated a rat homologue of the *Caenorrhabditis elegans lin-10* product. Although rat lin-10 is expressed in the cytosol and membrane fractions of various tissues, it is distributed only in the membrane fraction in brain where it is enriched in the synaptic plasma membrane and postsynaptic density fractions. We have isolated here a rat lin-10-interacting protein from rat brain and identified it to be neurabin-II/spinophilin, which has recently been isolated as a protein interacting with protein phosphatase I and F-actin. Neurabin-II/spinophilin is ubiquitously expressed but enriched in brain, especially in the synaptic plasma membrane and postsynaptic density fractions. We discuss the physiological significance of the interaction of rat lin-10 with neurabin-II/spinophilin.

In *C. elegans,* the vulval induction is initiated by a signal from the gonadal anchor cell to P6.p cell (1). In P6.p cell, a receptor tyrosine kinase/Ras signal transduction pathway, including the *lin-3, let-23,* and *let-60* products, is activated to cause the commitment of P6.p cell to the specific cell fate (2-7). *Lin-3* encodes an EGF-like protein that is the signal from the gonadal anchor cell (3,4). *Let-23* encodes the receptor for the *lin-3* product that is similar to the EGF receptor (5). *Let-60* encodes a Ras homologue which functions in the downstream of the *let-23* product (6, 7). In this pathway, the precise localization of the *let-23* product is important and determined by three other genes, *lin-2, lin-7,* and *lin-10. Lin-2* encodes a membrane-associated guanylate kinase (8) and *lin-7* encodes a small protein with one PDZ domain (9). *Lin-10* encodes a protein composed of 421 amino acids which has no significant homology to known genes (10).

In the preceding study, we have isolated a rat homologue of the *lin-10* product and named it rat lin-10 (11). Rat lin-10 is ubiquitously expressed. In most tissues, such as liver and lung, it is present in both the membrane and cytosol fractions, whereas it is localized only in the membrane fraction in brain, where it is enriched in the synaptic plasma membrane and postsynaptic density fractions. Since rat lin-10 does not have any hydrophobic region, we have assumed that rat lin-10 is associated with the membrane through some membrane protein in brain. On this assumption, we have screened here a rat brain cDNA library by use of the yeast two-hybrid method with rat lin-10 as a bait to isolate a rat lin10-interacting protein, and identified it to be neurabin-II/spinophilin.

Neurabin-II/spinophilin has been isolated independently by two laboratories as a protein interacting with PPI and F-actin (12,13). It is composed of 817 amino acids and has one PDZ domain and three coiled-coil regions. PPI binds to the region containing the amino acid residues 296 to 817. F-actin binds to the region containing the amino acid residues 1 to 154. Neurabin-II/spinophilin is considered to be a ubiquitously expressed isoform of neurabin-I, a neural specific F-actin binding protein, which has a domain organization similar to that of neurabin-II/spinophilin (14). The message of neurabin-II/spinophilin is detected in various tissues, but the protein is highly expressed in brain where it is enriched at synaptic junctions. The PDZ domain is a protein module which interacts mainly with trans-

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Abbreviations: *C. elegans, Caenorrhabditis elegans;* PPI, protein phosphatase I; MBP, maltose-binding protein; GST, glutathione-S-transferase; and GFP, green fluorescence protein.

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membrane proteins, such as receptors and channels (for review, see 15), and neurabin-II/spinophilin as well as neurabin-I is thought to bind some membrane protein at synaptic junctions through the PDZ domain. We describe here the interaction of rat lin-10 with neurabin-II/spinophilin and discuss the physiological significance of this interaction.

MATERIALS AND METHODS

Construction of expression vectors. Prokaryote and eukaryote expression constructs were constructed in pGexKG (16), pMalC2 (New England Biolabs), pCMV5 (a gift of David W. Russell), pEGFPN3 (Clontech), and pBTM116 using standard molecular biology methods (17). The following constructs of rat lin-10 contain the following amino acid residues: pBTM116 rat lin-10-2, 1-244; pBTM116 rat lin-10-3, 18-244; pBTM116 rat lin-10-4, 18-422; pEGFPN3 rat lin-10, 1-422; pCMV Myc rat lin-10, 1-422; and pMal rat lin-10, 1-422. The following constructs of neurabin-II/spinophilin contain the following amino acid residues: pGex neurabin-II-B, 1-154; pGex neurabin-II-C, 217-399; pGex neurabin-II-D, 771-817; pGex neurabin-II-F, 155-495; pGex neurabin-II-G, 584-817; pGex neurabin-II-H, 400-658; pCMV Myc neurabin-II-A, 1-817; pCMV Myc neurabin-II-B, 149-817; and pCMV Myc neurabin-II-C, 1-149.

Yeast two-hybrid screening. The construction of a rat brain cDNA prey library was described (18). The yeast two-hybrid screening of the library was performed using a mixture of three bait constructs (pBTM116 rat lin-10-2, -3, and -4) as described (18).

Cloning of rat lin-10-interacting protein. A rat brain cDNA library was purchased from Stratagene and the screening was performed as described (18). DNA sequencing was performed by the dideoxy nucleotide termination method using an ABI373 DNA sequencer.

Antibodies. A rabbit polyclonal antibody was raised against the synthetic peptide containing the amino acid residues 386-404 of rat lin-10, and purified on HiTrap NHS-activated affinity column coupled with the peptide. A mouse monoclonal antibody was raised against GST-neurabin-II-G and the medium of hybridoma cells were used. The monoclonal antibody against the myc tag, MYC 1-9E10.2, was obtained from American Type Culture Collection.

In vitro binding of GST-fusion neurabin-II/spinophilin with rat lin-10 expressed in COS cells. COS cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and transfected with pCMV Myc rat lin-10 using the DEAE-dextran method. Cells from one 10-cm dish were homogenized in 0.5 ml of 20 mM Hepes/NaOH at pH 8.0 containing 0.15 M NaCl, 1 mM EDTA, and 1% (w/v) Triton X-100, followed by centrifugation at 100,000 × g for 30 min at 4°C to collect the supernatant. 0.5 ml of the supernatant was incubated with 100 pmol of various GST-constructs of neurabin-II/spinophilin fixed on glutathione beads. After incubation for 4 hr at 4°C, the beads were washed and the associated proteins were detected by immunoblotting with the anti-myc antibody.

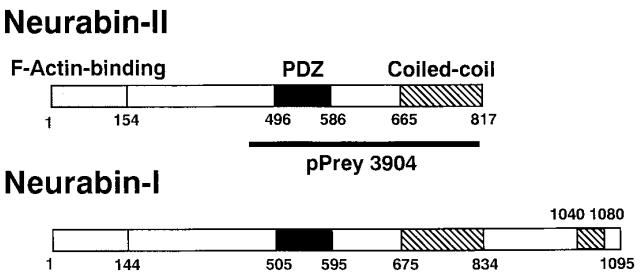

FIG. 1. Schematic domain structures of neurabin-II/spinophilin and neurabin-I. Numbers indicate the numbers of the amino acid residues. F-Actin binding domains are shaded. PDZ domains are black. Coiled-coil regions are hatched. The black bar shows the region which pPrey 3904 clone from the yeast two-hybrid screening contains.

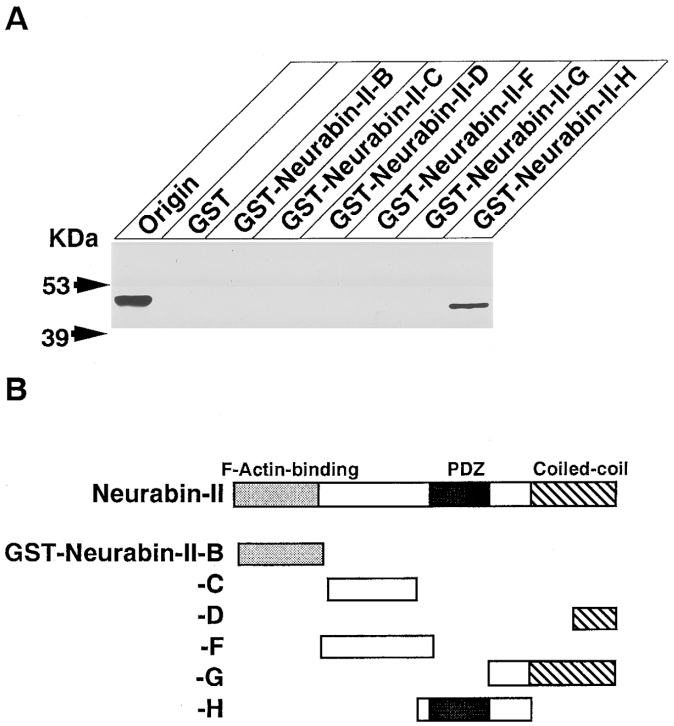

FIG. 2. Binding of rat lin-10 with neurabin-II/spinophilin. (A) Binding of the myc-tagged rat lin-10 to GST-neurabin-II-H. The extracts of COS cells expressing the myc-tagged rat lin-10 were incubated with various GST-constructs of neurabin-II/spinophilin fixed on glutathione beads. The associated proteins were detected with the anti-myc antibody. GST-constructs are indicated on the *top*. GST is a control GST protein. (B) GST-constructs of neurabin-II/spinophilin. Bars indicate the regions contained in various GST-constructs.

Surface plasmon resonance measurement of interaction of rat lin-10 with neurabin-II/spinophilin. 200 pmol of GST-neurabin-II-H, -C, and GST were separately fixed on a CM5 research grade sensor chip through the antibody against GST, and the MBP-fusion proteins of rat lin-10 or SNAP-25A were superfused at 3.5 to 21 μM. Data were analyzed with the BIA Evaluation program 2.1 (19).

Transfection and immunocytostaining of HEK293 cells. HEK293 cells were grown in Dulbecco's modified Eagle medium supplemented with 10 % horse serum, and transfected with pEGFPN3 rat lin-10 with various pCMV Myc constructs of neurabin-II/spinophilin using the calcium phosphate method and immunostained with the anti-myc antibody.

Miscellaneous procedures. The primary cultures and immunocytochemistry of rat hippocampal neurons were performed using a confocal imaging system (Bio-Rad MRC1024) as described (20). SDS-PAGE, Western blotting, and protein determination were performed using standard procedures as described (20).

RESULTS AND DISCUSSION

We screened more than six million clones of the rat brain cDNA prey library with a mixture of three bait constructs containing various regions of rat lin-10. Four clones, pPrey 3901, pPrey 3902, pPrey 3904, and pPrey 3930, were isolated and confirmed to interact with pBTM116 rat lin-10-2 containing the N-terminal region of rat lin-10. pPrey 3904 encoded a PDZ domain and we focused on this clone. Analysis of other clones will be described elsewhere. To obtain a full length clone, we screened four million clones of the rat brain cDNA library with a probe prepared from pPrey 3904 and obtained more than 60 positive clones. Twenty nine clones were recovered and four overlapping clones were closely analyzed. One clone among them, p3904-4, contained a 2.4-kb open reading frame and the putative initiator methionine which agreed well with the consensus sequence of initiator codons (21). This gene encoded a protein composed of 817 amino acids which predicted a hydrophilic protein without a transmembrane region or a signal sequence. One PDZ domain existed in the middle region. The product of this gene turned out to be neurabin-II/spinophilin (12,13). pPrey 3904 contained the amino acid residues 430-817 of neurabin-II/spinophilin (Fig. 1).

We confirmed the interaction of rat lin-10 with neurabin-II/spinophilin using three methods other than yeast two-hybrid method. Firstly, we tested the GST-constructs containing various regions of neurabin-II/spinophilin fixed on glutathione beads for the interaction with rat lin-10 expressed in COS cells. Only the GST-neurabin-II-H containing the amino acid residues 400-658 of neurabin-II/spinophilin interacted with rat lin-10 (Fig. 2).

Secondly, we examined the interaction of rat lin-10 with neurabin-II/spinophilin by use of the surface plasmon resonance method. The GST-constructs of neurabin-II/spinophilin were fixed on the BIAcore sensor chip through the antibody against GST. The MBP-fusion protein containing full length rat lin-10 (MBP-rat lin-10) was flowed on the chip. MBP-rat lin-10 bound to GST-neurabin-II-H, but not to GST-neurabin-II-C or GST (data not shown). The MBP-fusion protein containing SNAP-25A did not bind any GST-fusion protein (data not shown). The K_D value of rat lin-10 for the GST-fusion protein was calculated to be $1.2 \pm 0.3 \times 10^{-6}$ M from the association (3.2×10^{3} $M^{-1}S^{-1}$) and dissociation (4.1×10^{-3} S^{-1}) constants.

Thirdly, we examined the interaction of rat lin-10 with neurabin-II/spinophilin in intact cells. The GFP-fusion protein of rat lin-10 (GFP-rat lin-10) and various regions of neurabin-II/spinophilin with the myc-tag at the N-terminus were expressed in HEK293 cells (Fig. 3A). GFP-rat lin-10 was distributed in the cytosol when expressed alone (Fig. 3B). Full-length neurabin-II/spinophilin with the myc-tag (the product of pCMV Myc neurabin-II-A) was distributed in the membrane (Fig. 3C). The products of the constructs of neurabin-II/spinophilin lacking the N-terminal region (pCMV Myc neurabin-II-B) and containing only the N-terminal region (pCMV Myc neurabin-II-C) were also distributed in the membrane (data not shown). When GFP-rat lin-10 was coexpressed with the product of either pCMV Myc neurabin-II-A or -B, GFP-rat lin-10 was translocated to the membrane and colocalized with the product of either pCMV Myc neurabin-II-A or -B (Fig. 3, D and E). However, when GFP-rat lin-10 was expressed with the product of pCMV Myc neurabin-II-C, GFP-rat lin-10 did not translocate to the membrane (Fig. 3F).

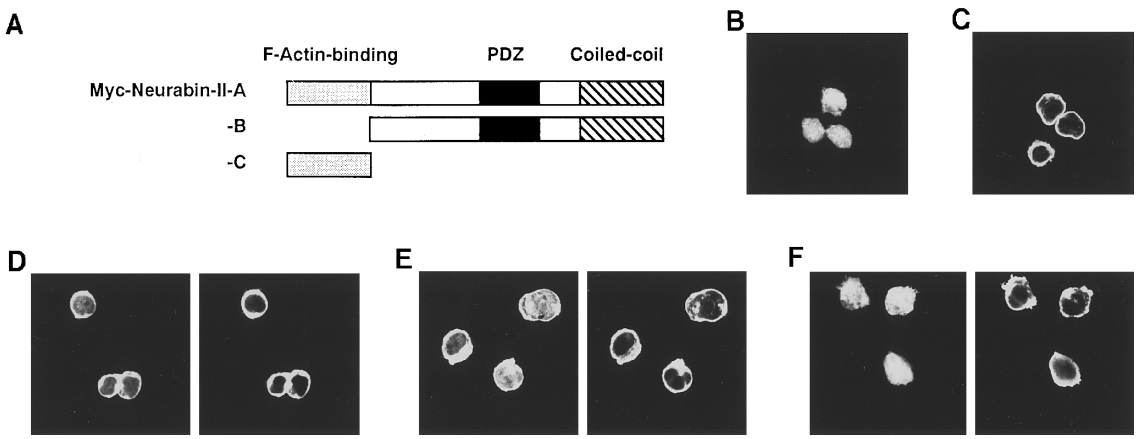

FIG. 3. Interaction of rat lin-10 with neurabin-II/spinophilin in HEK293 cells. HEK293 cells were transfected with pEGFPN3 rat lin-10 and various pCMV Myc neurabin-II constructs. The signal of GFP was obtained at 416 nm and the products of pCMV Myc constructs were stained with the anti-myc and Cy5-conjugated anti-mouse IgG antibodies. (A) Schematic descriptions of pCMV Myc neurabin-II-A, -B, and -C. (B) GFP-rat lin-10 expressed alone. (C) Myc-neurabin-II-A expressed alone. (D) GFP-rat lin-10 (left) and Myc-neurabin-II-A (right). (E) GFP-rat lin-10 (left) and Myc-neurabin-II-B (right). (F) GFP-rat lin-10 (left) and Myc-neurabin-II-C (right).

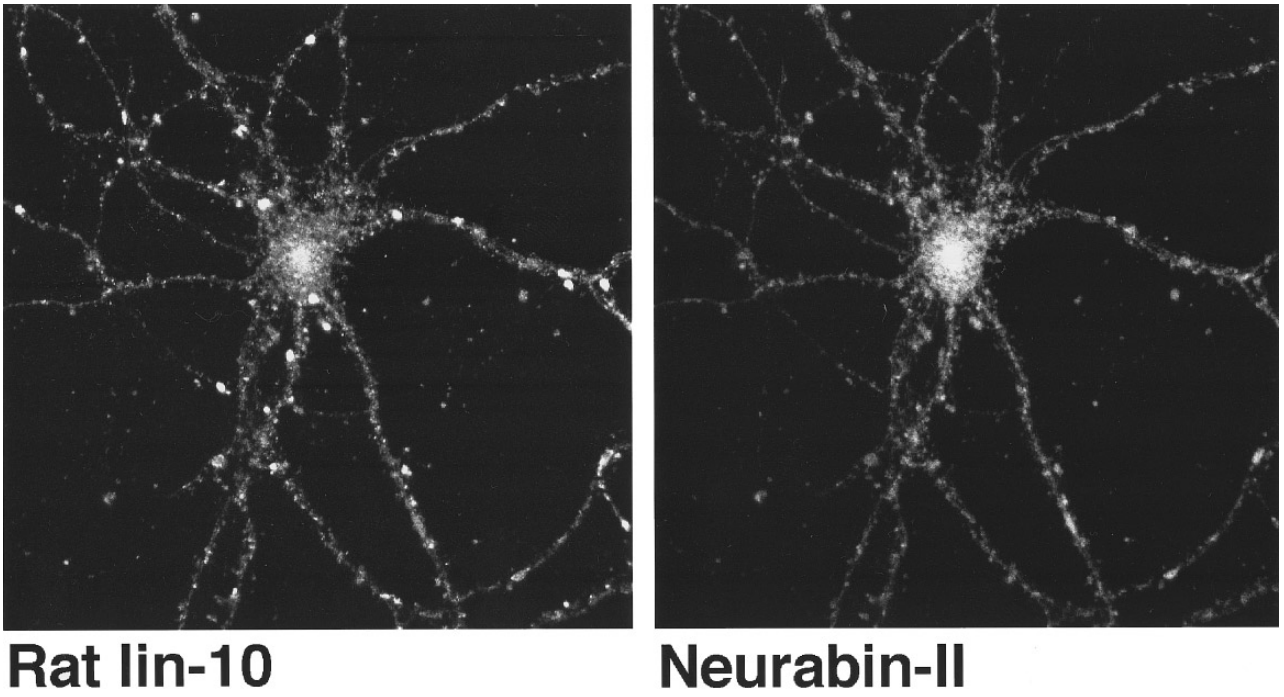

FIG. 4. Expression of rat lin-10 in primary cultured rat hippocampal neuron. Rat hippocampal neurons were cultured and double-stained with the polyclonal anti-rat lin-10 and monoclonal anti-neurabin-II antibodies. Rat lin-10 (left) and neurabin-II/spinophilin (right).

Therefore, rat lin-10 interacted with neurabin-I-I/spinophilin in intact cells, and the N-terminal region of neurabin-II/spinophilin was not necessary for the interaction. From the data shown in Fig. 2, the interacting domain of rat lin-10 with neurabin-II/spinophilin was mapped in the middle region containing the PDZ domain. The amino acid sequence of the PDZ domain of neurabin-II/spinophilin has 86 % homology to that of neurabin-I (13). However, rat lin-10 was not colocalized with neurabin-I in HEK293 cells (data not shown). These findings suggest that rat lin-10 interacts with neurabin-II/spinophilin via not only the PDZ domain but also some other region.

Finally, we checked whether rat lin-10 was colocalized with neurabin-II/spinophilin in primary cultured rat hippocampal neurons. Rat hippocampal neurons were double-stained with the polyclonal antibody against rat lin-10 and the monoclonal antibody against neurabin-II/spinophilin. Rat lin-10 was colocalized with neurabin-II/spinophilin mainly in the dendrites (Fig. 4).

Cortical microfilaments are involved in many membrane-based cellular events including signal transduction triggered by extracellular signals besides cell migration, cytokinesis, and membrane transport. Recent studies have revealed that receptors for extracellular signals are localized at submembranous domains which are attached with the actin cytoskeleton (for review, see 22). This linkage of the actin cytoskeleton with the plasma membrane is mediated by various proteins. Neurabin-II/spinophilin may interact with some unidentified membrane receptor through the PDZ domain and link it to the actin cytoskeleton. If the interaction of neurabin-II/spinophilin with rat lin-10 affects the interaction with such a membrane receptor, rat lin-10 could regulate the linkage of the receptor with the actin cytoskeleton and be involved in the localization of the receptor. This model is concordant with that the *lin-10* product is involved in the localization of the *let-23* product, a membrane tyrosine kinase receptor in *C. elegans* (10). To verify this model, it is essential to identify a neurabin-II/spinophilin-interacting membrane protein and it is also helpful to identify a *C. elegans* homologue of neurabin-II/spinophilin and check whether such a homologue is involved in the localization of the *let-23* product.

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