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# Identification of a cell polarity-related protein, Lin-7B, as a binding partner for a Rho effector, Rhotekin, and their possible interaction in neurons

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**Abstract**

Rhotekin, an effector of Rho, is highly expressed in the brain but its function(s) in neurons is almost unknown. In an attempt to define the properties of Rhotekin in neuronal cells, we focused on its interaction with polarity-related molecules. In the present study, we identified a PDZ protein, Lin-7B, as a binding partner for Rhotekin by yeast two-hybrid screening of human brain cDNA library. We then found that Rhotekin interacts with Lin-7B in in vitro pull-down assays, and forms an immunocomplex in COS7 cells and the rat brain. The C-terminal three amino acids of Rhotekin were essential for the interaction with Lin-7B. Their binding affinity became increased in the presence of active RhoA in the COS7 cell expression system. In addition, immunohistochemical analyses demonstrated that Lin-7 as well as Rhotekin is enriched in neurons. These results suggest that Lin-7 plays some role in neuronal functions in concert with Rho/Rhotekin signals.


*Keywords:* Rho; Rhotekin; Lin-7; Synapse; Neuronal cell polarity

## 1. Introduction

The small GTPase Rho is known to play important roles in various cellular functions such as actin cytoskeletal reorganization, transcriptional activation, tumor cell invasion, cell morphology, cell motility and cytokinesis (Jaffe and Hall, 2002). There has been much progress in elucidating molecular mechanisms of Rho-dependent cellular processes such as actin reorganization (Ridley, 2001; Fukata et al., 2003). Rho also regulates various neuronal cellular processes such as neurite retraction and neuronal polarization (Negishi and Katoh, 2002; Govek et al., 2005).

A variety of Rho effector molecules have so far been identified and shown to play pivotal roles in the Rho-dependent neuronal cellular events (Bito, 2003). Rhotekin, which is highly expressed in the brain, is one of the Rho effectors (Reid et al., 1996). In contrast to the accumulated information about the functions of several Rho effectors, our knowledge about the physiological significance of Rhotekin is very limited. Rhotekin has been reported to control Rho-dependent gene transcription and septin filament structures, although the precise molecular mechanisms of these events remain to be clarified (Reynaud et al., 2000; Liu et al., 2004; Ito et al., 2005). Presence of pleckstrin-homology (PH) domain and two proline-rich motifs towards the C-terminus (Fig. 1A) strongly suggests physiological roles of Rhotekin through protein–protein interactions. However, molecules interacting with Rhotekin are largely unknown. It is notable that Rhotekin exhibits at the C-terminus the sequence QSPV that matches the X(S/T)XV consensus known for proteins recognizing PDZ (PSD-95, Discs-large and ZO-1) domains. The PDZ domain is known to be present in a rapidly increasing number of proteins exhibiting diverse functions. Many proteins binding to this domain are thought to play important roles in cell polarity, cell adhesion and cell signaling (Sheng and Sala, 2001). Therefore, Rhotekin might exert the functions in cell polarity

*Abbreviations:* PH, pleckstrin-homology; RBD, active Rho-binding domain; GST, glutathione *S*-transferase; aa, amino acid; *E. coli*, *Escherichia coli*; RNAi, RNA interference; LPA, lysophosphatidic acid

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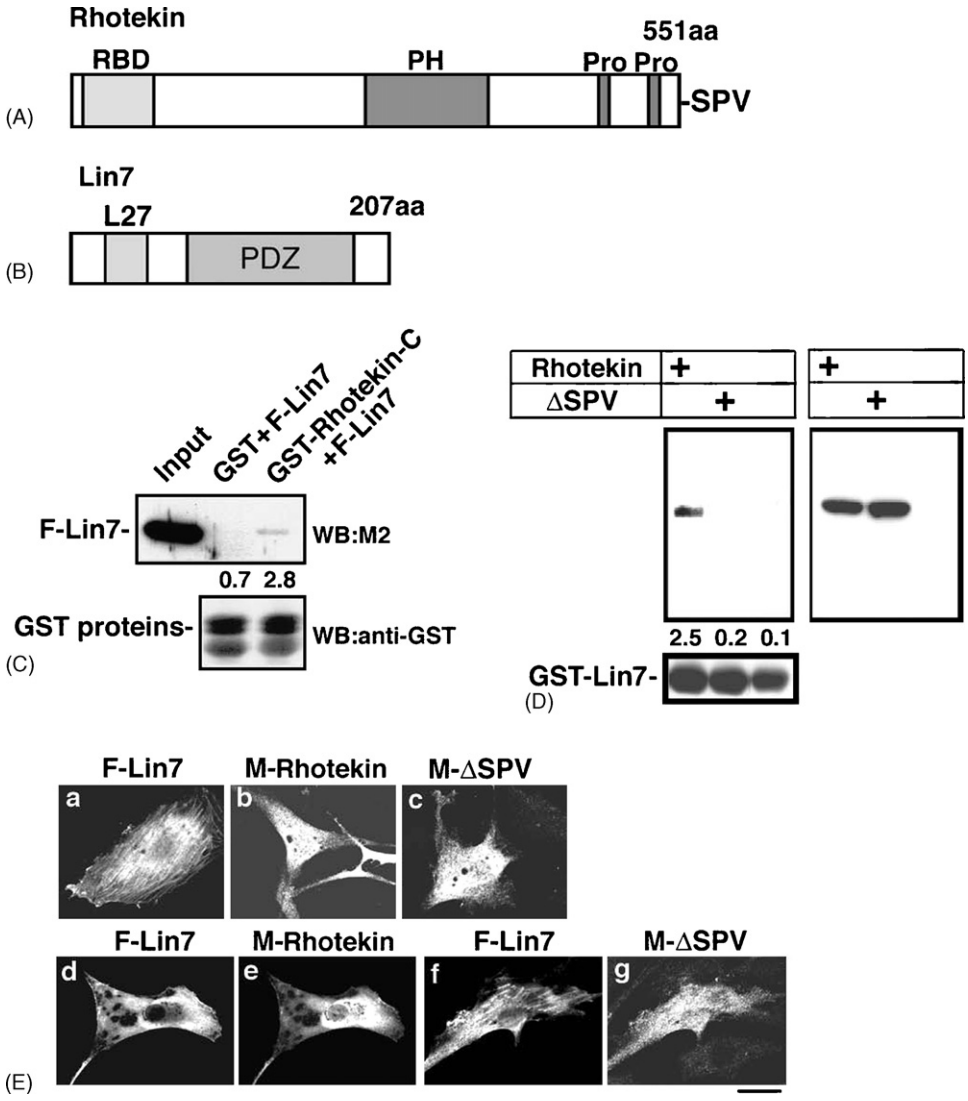


Fig. 1. Interaction of Lin-7B with Rhotekin. (A and B) Domain cartoons of Rhotekin (A) and Lin-7B (B). Structural domains of the protein are abbreviated as follows: RBD, Rho-binding domain; PH, pleckstrin-homology; Pro, proline-rich motif; L27, heterodimerization domain in Lin-2 and Lin-7. (C) Co-sedimentation of Lin-7B with GST-Rhotekin-C. COS7 cell lysate expressing Flag-Lin-7B (90% of total protein amounts) was incubated with 3 μg of GST or GST-Rhotekin-C followed by pull-down with glutathione-agarose beads. Twenty percent of the resultant samples and the original lysate (input; 5% of the total protein amounts) were separated by SDS-PAGE (15% gel) followed by Western blotting with M2 (upper panel) or anti-GST (lower panel) to detect Flag-Lin-7B and GST-proteins, respectively. (D) Co-sedimentation of Flag-Rhotekin and -RhotekinΔSPV with GST-Lin-7B. COS7 cell lysate expressing Flag-Rhotekin or -RhotekinΔSPV (90% of total protein amounts) was incubated with 3 μg of GST or GST-Lin-7B followed by pull-down with glutathione-agarose beads. Twenty percent of the resultant samples (left upper panel) and the original lysates (right panel; 5% of the total protein amounts) were separated by SDS-PAGE (10% gel) followed by Western blotting with M2. The amounts of precipitated GST-Lin-7B in each sample were detected by re-probing the blot with anti-GST (left lower panel). (C and D) Relative levels of Flag-Lin-7B or Rhotekin in the precipitates were measured by NIH Image software and expressed as percentages to the total amount in the lysate used in each assay. (E) Effects of Rhotekin or RhotekinΔSPV on the localization of Lin-7B. REF52 cells expressing Flag-Lin-7B (a), Myc-Rhotekin (b) or Myc-RhotekinΔSPV (c) were stained with M2 (a) or 9E10 (b and c). Cells expressing Flag-Lin-7B with Myc-Rhotekin (d and e) or Myc-RhotekinΔSPV (f and g) were double-stained with M2 (d and f) and polyclonal anti-Myc (e and g). Bar, 20 μm.

regulation through interactions with yet unidentified PDZ proteins. In this context, a PDZ protein, PDZ domain protein interacting specifically with TC10 (PIST), which is involved in protein trafficking in synapses (Cuadra et al., 2004), was just identified as a binding partner for Rhotekin (Ito et al., 2006).

In the present study we identified Lin-7B (GenBank accession number NM_022165), also termed as Veli-2 and MALS-2 (Butz et al., 1998; Irie et al., 1999; Jo et al., 1999), as an interactive partner for Rhotekin, using yeast two-hybrid screening with human brain library. Lin-7 appears to be involved in synapse functions since it is reported to associate with the neuronal cadherin-β-catenin complex (Perego et al., 2000). We found that Rhotekin interacts with Lin-7 in the brain tissues, and that Lin-7 and Rhotekin are abundant in the brain and distributed in neurons at thalamus, hippocampus CA1 region and dentate gyrus. Since Lin-7 as well as Rhotekin is enriched at synapses in rat primary cultured hippocampal neurons, these two proteins are likely to be involved in neuronal cell polarity formation and/or maintenance possibly in a concerted manner.

## 2. Materials and methods

### 2.1. Plasmid construction

Rhotekin was a kind gift from Dr. S. Narumiya (Kyoto University, Japan). pRK5 Flag or Myc vectors harboring human RhoA, Rac1, Cdc42 and C3 exoenzyme are from Dr. Alan Hall (University College London). Rhotekin, RhotekinΔSPV lacking C-terminal SPV motif, RhotekinΔRBD lacking active Rho-binding domain (aa 89–551), Rhotekin-C (aa 513–551) and Lin-7B were subcloned into pRK5-Myc, pRK5-Flag, pYTH9 or pGEX-4T3 vector. For RNAi experiments, two single-stranded DNA oligonucleotides (top strand, TGCTGTGATGTTGAA GCCTAGGCCCTGTTTTGGC-CACTGACTGACAGGGCCTACTTCAACATCA; bottom strand, CCTGTG-ATGTTGAAGTAGGCCCTGTCAGTCAGTGGCC AAAACAGGGCCTAG-GCTTCAACATCAC) were designed, annealed and cloned into pcDNA6.2-GW/EmGFP-miR vector (Invitrogen). All constructs were verified by DNA sequencing.

### 2.2. Yeast two-hybrid analyses

pYTH9-Rhotekin-C was used as a bait (Fig. 1A) in the two-hybrid screen with human brain cDNA library fused to pACT2 (BD Biosciences Clontech). Subsequent two-hybrid interaction analyses were carried out as described (Nagata et al., 1998).

### 2.3. In vitro co-sedimentation assays

GST, GST-Rhotekin-C, GST-Lin-7B were expressed in *Escherichia coli* and affinity-purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech). COS7 cell lysates expressing Flag-Lin-7B, -Rhotekin or -RhotekinΔSPV were incubated with GST-Rhotekin-C or GST-Lin-7B. GST was also used as a control. Pull-down assays were done as described (Ito et al., 2006). SDS-PAGE and Western blot analysis was done as described (Nagata et al., 2003).

### 2.4. Antibodies

Using GST-Lin-7B expressed in *E. coli* as an antigen, a rabbit polyclonal antibody specific for Lin-7B was generated and affinity-purified on a column to which the antigen had been conjugated. Anti-Rhotekin antibody produced in our laboratory was described previously (Ito et al., 2005). Polyclonal anti-Flag and monoclonal anti-Flag M2 antibodies were from Kodak. Polyclonal anti-Myc and monoclonal anti-Myc 9E10 were from Santa Cruz Biotech. Monoclonal antibodies for synaptophysin and β-tubulin were from Sigma–Aldrich. Monoclonal anti-ZO-1 was from Chemicon.

### 2.5. Preparation of rat brain extracts and Western blotting

To analyze the regional expression of Lin-7 in rat brain, whole extracts were prepared with 50 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaF, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2% SDS. SDS-PAGE and Western blotting was performed as described (Nagata and Inagaki, 2005). Concentrations of protein were estimated with a micro BCA protein assay reagent kit (Pierce) with bovine serum albumin as the standard.

### 2.6. Cell culture, transfection and immunofluorescence

Rat hippocampal neurons were cultured essentially as described (Goslin et al., 1998). Transient transfection was carried out using the Lipofectamine method (Gibco-BRL). Immunofluorescence analysis was done as described (Nagata et al., 2004). Anti-Lin-7 and anti-synaptophysin, a synaptic marker, were used as primary antibodies. Alexa Fluor 488- or 568-labeled IgG (Molecular Probes) was used as a secondary antibody. Fluorescent images were obtained using a FLUOVIEW confocal microscope (OLYMPUS).

### 2.7. Immunoprecipitation

Immunoprecipitation was done as previously described (Nagata and Inagaki, 2005). Briefly, COS7 cells (35 mm dish, $1 \times 10^4$ cells/dish) expressing tagged proteins, or adult rat brains were homogenized with lysis buffer containing 40 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.1 M NaF, 100 μM $Na_3VO_4$, 0.5% NP-40, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Insoluble material was removed by centrifugation at 4 °C for 60 min at 100,000 × *g*, and the lysate was used for each assay. Immunoprecipitation was carried out with M2 or anti-Lin-7 antibody for COS7 cell expression analyses or anti-Rhotekin for detecting endogenous Rhotekin–Lin-7 complex.

### 2.8. Immunohistochemical studies

Rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The fixed brains were embedded in paraffin, cut into 6 μm thick subserial coronal sections at the level of dorsal hippocampus. After deparaffinization, sections were processed for immunohistochemistry as reported previously (Shinohara et al., 1998). Histochemical images were obtained using BX61 microscope with an attached DP-70 digital camera (OLYMPUS).

## 3. Results

### 3.1. Identification of Lin-7B as a binding partner for Rhotekin

In order to search for candidate proteins involved in the Rho/Rhotekin signaling, we attempted to identify binding partners for Rhotekin. We focused on the C-terminus of the protein, since it contains a consensus binding motif for the class I PDZ domain, and thus possible to be involved in cell polarity, cell adhesion and cell signaling (Sheng and Sala, 2001). We performed a yeast two-hybrid screen with Rhotekin-C as a bait and a cDNA library from human brain (Fig. 1A). After screening of $2.0 \times 10^5$ clones, we obtained 83 positive clones. Partial cDNA sequence analyses revealed that four clones showing a strong positive interaction with Rhotekin-C corresponded to Lin-7B also known as MALS-2 or Veli-2 (Fig. 1B) (Butz et al., 1998; Irie et al., 1999; Jo et al., 1999). Lin-7 contains a PDZ domain and a heterodimerization domain, L27 (Doerks et al., 2000).

It is most likely that Rhotekin binds to the PDZ domain of Lin-7B through the C-terminus SPV motif. To confirm the interaction between the C-terminus of Rhotekin with Lin-7B, we performed pull-down analyses. A purified GST-Rhotekin-C or GST was incubated with COS7 cell lysates expressing Flag-Lin-7B, and the resultant complexes were separated on SDS-PAGE followed by Western blotting with M2. As shown in Fig. 1C, GST-Rhotekin-C was capable of binding Flag-Lin-7B under the conditions used. We also tested if GST-Lin-7B can pull-down Rhotekin in the C-terminal motif-dependent manner. As shown in Fig. 1D, GST-Lin-7B precipitates Flag-Rhotekin but not RhotekinΔSPV lacking the SPV motif, confirming the importance of the C-terminal amino acid motif for Rhotekin–Lin-7B interaction. We next examined localization of Lin-7B and Rhotekin overexpressed in rat embryonic fibroblast REF52 cells. As shown in Fig. 1E(a–c), Flag-Lin-7B was distributed in a filamentous manner while Myc-Rhotekin

and -RhotekinΔSPV were localized diffusely in the cytoplasm. When Flag-Lin-7B and Myc-Rhotekin were co-expressed, Flag-Lin-7B came to show cytoplasmic diffuse localization (Fig. 1E, d and e). The altered Lin-7B localization is likely to be caused by the interaction with cytosolic Myc-Rhotekin. In contrast, filamentous localization of Flag-Lin-7B was still observed when co-expressed with Myc-RhotekinΔSPV, suggestive of no interaction between Lin-7B and the Rhotekin mutant (Fig. 1E, f and g). Based on these results we concluded that Rhotekin interacts with the PDZ domain of Lin-7 through the C-terminal SPV motif.

### 3.2. Interaction of Rhotekin with Lin-7B in COS7 cells

The data obtained by two-hybrid analyses and pull-down assays strongly suggest that Rhotekin and Lin-7B form a complex in cells. To test this possibility, immunoprecipitation analyses were carried out using the COS7 cell transient expression method. As shown in Fig. 2A, Flag-Lin-7B was co-immunoprecipitated with Myc-Rhotekin.

Since Rhotekin is a target protein for Rho, we asked if Rhotekin–Lin-7B interaction is influenced by Rho family of small GTPases. As shown in Fig. 2B, constitutively activated versions of RhoA (L63Rho) increased the binding affinity between Rhotekin and Lin-7B in COS7 cells. On the other hand, Rac (L61Rac) and Cdc42 (L61Cdc42) had little effects on the interaction (Fig. 2B). Apparently low increase of Flag-Lin-7B and Myc-Rhotekin binding may be due to the basal Rho activity in COS7 cells, which facilitates Lin-7B-Rhotekin interaction to some extent. We thus asked if C3 exoenzyme inhibits the co-immunoprecipitation of Flag-Lin-7B and Myc-Rhotekin. As shown in Fig. 2C, expression of Myc-C3 had no effects on the interaction. Dominant negative forms of Rho (N19Rho), Rac (N17Rac) and Cdc42 (N17Cdc42) also had no effects on their interaction (data not shown). We also asked if lysophosphatidic acid (LPA) treatment facilitates Lin-7B-Rhotekin binding through activation in endogenous Rho, but consequently LPA had little effect on the interaction (Fig. 2C). Since active Rho increased the interaction between Lin-7B and Rhotekin in COS7 cells, we further tried to examine the effects of active Rho on the interaction between Lin-7B and RhotekinΔRBD. Surprisingly, RhotekinΔRBD was no longer co-immunoprecipitated with Lin-7B under the condition used (Fig. 2D). We assume that the result is due to the conformational change by the deletion of RBD. Taken together, these results suggest that the Rhotekin–Lin-7B interaction is dependent on Rho activity, although precise molecular mechanism(s) regulating their interaction remains to be elucidated.

### 3.3. Production of anti-Lin-7 antibody and detection of Lin-7 in various rat tissues and brain regions

To further characterize Lin-7, we developed a rabbit polyclonal antibody (anti-Lin-7) against the bacterially synthesized Lin-7B. Specificity of the affinity-purified antibody was confirmed by Western blot analyses using lysate from COS7 cells expressing Lin-7B. As shown in Fig. 3A (upper left panel), we detected Flag-Lin-7B with an apparent molecular mass of 26 kDa in the lysate. Preincubation of the antibody with the antigen inhibited the immunoreactivity (Fig. 3A, upper right panel). Since anti-Lin-7 was raised against full length human Lin-7B which contains the conserved regions among Lin-7A/MALS-1/Veli-1, -7B and -7C/MALS-3/Veli-3, this antibody is considered to recognize the three Lin-7 isoforms. The immunoreactivity was markedly reduced when Flag-Lin-7B expression was suppressed by co-transfection of pcDNA6.2-GW/EmGFP-miR-Lin-7, which expresses microRNA for RNAi of Lin-7 (Fig. 3B). We next did a Western blot analysis to detect endogenous Lin-7 in lysates of mammalian cell lines, including C6 glioma, Madin–Darby Canine Kidney (MDCK), fibroblast REF52 and differentiated neuroblastoma Neuro2A cells (Fig. 3C). In C6, MDCK and Neuro2A cells, an immunopositive band with molecular mass of ∼25 kDa was observed. Slightly high molecular mass of Flag-Lin-7B might be due to the attached Flag-tag. Although it is difficult to conclude which band corresponds to each isoform, we assume the 25 kDa protein is Lin-7B and/or Lin-7C based on the previous study where these two isoforms were assigned to 25 kDa (Jo et al., 1999). We also examined intracellular localization of Lin-7 in MDCK, REF52 and Neuro2A cells by immunofluorescent analyses. In MDCK cells, Lin-7 was co-localized with a tight junction marker, ZO-1, at cell–cell contact sites (Fig. 3D, a and b). In REF52 cells, Lin-7 was distributed at perinuclear and marginal regions, and slightly with stress fibers (Fig. 3D, c and d). As for Neuro2A cells, Lin-7 was distributed throughout the cytoplasm and also enriched at the neurite tip (Fig. 3I, e). This localization pattern is similar to that of Rhotekin (Ito et al., 2005).

We next did Western blot analyses to detect endogenous Lin-7 in lysates from a variety of rat tissues, and detected proteins with apparent molecular masses of 25 and 28 kDa (Fig. 4A). We assume that the 25 and 28 kDa proteins are Lin-7B/C (MALS-2/3) and Lin-7A (MALS-1), respectively, based on their molecular masses and a previous report (Jo et al., 1999). The 25 kDa protein was dominantly present in brain tissues but also detected in many tissues while the 28 kDa protein was specifically expressed in brain tissues (Fig. 4A). A previous report claims that Lin-7 proteins are expressed only in rat brain, heart and kidney (Jo et al., 1999). The reason of the discrepancy is not clear but we assume that Lin-7 is possible to express in many tissues and regulates cell polarity as described in epithelial cells (Yamamoto et al., 2002). Lin-7 proteins were generally present as membrane-bound forms in many rat tissues in our analyses. A 19 kDa isoform was detected as a membrane protein strongly and weakly in long extensor muscle (EDL) and soleus muscle (SOL), respectively. It is not clear if this molecule is a muscle-specific isoform of Lin-7 or a degradation product.

To characterize further the expression of Lin-7 proteins in the brain, thirteen regions were dissected from adult rats and subjected to Western blot analyses using anti-Lin-7 antibody (Fig. 4B, upper panel). The results show that Lin-7 is relatively abundant in cerebellum and the telencephalon, including

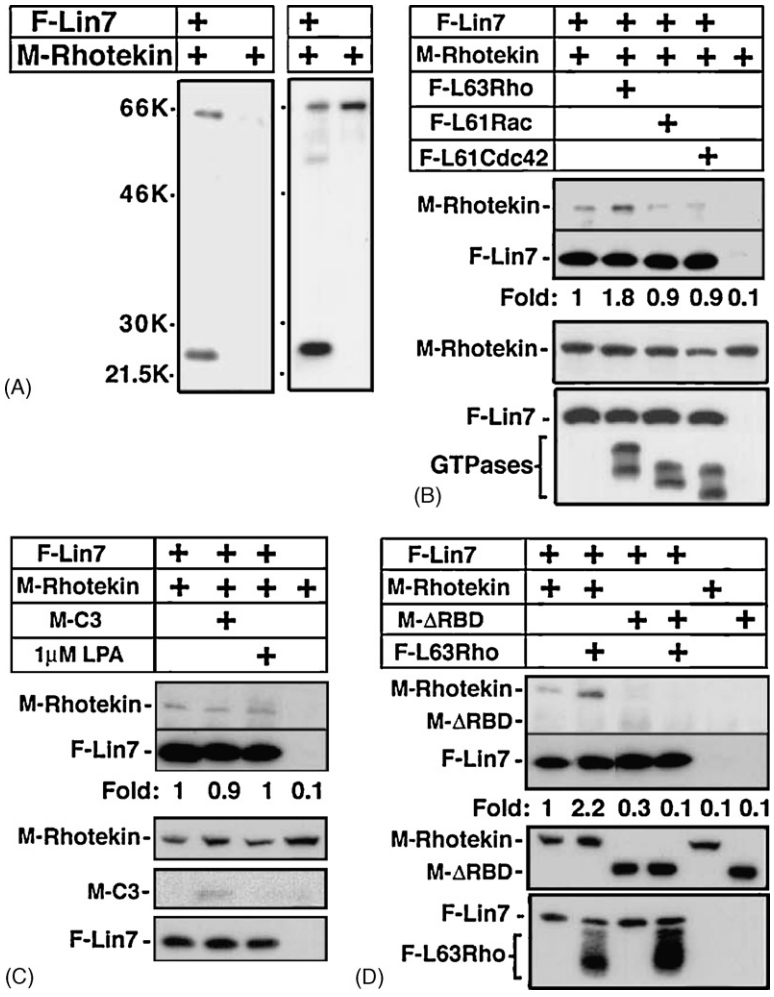


Fig. 2. Immunocomplex formation of Lin-7B with Rhotekin in COS7 cells. (A) Co-immunoprecipitation analyses of Flag-Lin-7B with Myc-Rhotekin. Lysates from transfected cells (90% of total protein amounts) were immunoprecipitated with M2 as described in Section 2. Twenty percent of the precipitated materials were subjected to SDS-PAGE (10% gel) followed by Western blotting with a mixture of polyclonal anti-Flag and anti-Myc antibodies (left panel). Expression of each protein was confirmed by Western blotting of cell lysates (3% of total volume) with a mixture of M2 and 9E10 (right panel). (B) Effects of activated versions of Rho, Rac and Cdc42 on the interaction between Rhotekin and Lin-7B. Cells were transfected with pRK5-Myc-Rhotekin, pRK5-Flag-Lin-7B, -L63RhoA, -L61Rac1 and -L61Cdc42 in various combinations. Lysates from transfected cells (90% of total protein amounts) were immunoprecipitated with anti-Lin-7 antibody. Western blotting was carried out with a mixture of 9E10 and M2 to detect Myc-Rhotekin and Flag-Lin-7B, respectively (upper two panels). Relative levels of Myc-Rhotekin in the immunopellets were measured with NIH Image software. Expression of each protein was confirmed by Western blotting with a mixture of M2 and 9E10 (lower two panels). (C) Effects of C3 exoenzyme and LPA treatment on the interaction between Rhotekin and Lin-7B. Cells were transfected with pRK5-Myc-Rhotekin, -Myc-C3 and pRK5-Flag-Lin-7B in various combinations and treated with 1/vM LPA for 30 min or left untreated. Lysates from transfected cells were immunoprecipitated and Western blotting was done as in (B) (upper two panels). Relative levels of Myc-Rhotekin in the immunopellets were also shown. Expression of each protein was confirmed as in (B) (lower three panels). (D) Effects of activated Rho on the interaction between RhotekinΔRBD and Lin-7B. Cells were transfected with pRK5-Myc-Rhotekin, -RhotekinΔRBD, pRK5-Flag-Lin-7B and -L63RhoA in various combinations. Immunoprecipitation and Western blotting was done as in (B) (upper two panels). Expression of each protein was confirmed as in (B) (lower two panels).

hippocampus, neocortex, entorhinal cortex and visual cortex. Lin-7 proteins were expressed moderately in other regions except the brain stem where only weak band with 25 kDa was detected. As a control experiment, synaptophysin distribution pattern was also analyzed and found to be very similar as described previously (Fig. 4B, lower panel) (Chin et al., 2000). The enrichment of Lin-7 in the telencephalon suggests that this protein may have an essential role in the neuronal function such as synapse formation and maintenance. We then examined subcellular distribution of Lin-7 with anti-Lin-7 in neurons. As shown in Fig. 4C(a–f), Lin-7 was found to co-localize with a synaptic marker, synaptophysin, in rat primary cultured hippocampal neurons. This is consistent with the previous data (Jo et al., 1999), confirming the quality of anti-Lin-7 we made. Rhotekin was also co-localized with synaptophysin (Fig. 4C, g–i), suggesting interaction of Rhotekin with Lin-7 at synapses.

The localization profiles of Lin-7 and Rhotekin in the brain were then determined by immunohistochemistry. Tissue sections from the adult rat brain were immunostained with anti-Lin-7 or anti-Rhotekin. Immunostaining of Lin-7 was observed in perikarya and axon/dendrite of neurons in thalamus (Fig. 5A), hippocampus CA1 region (Fig. 5B) and dentate gyrus (Fig. 5C). Although the distribution pattern of Rhotekin is

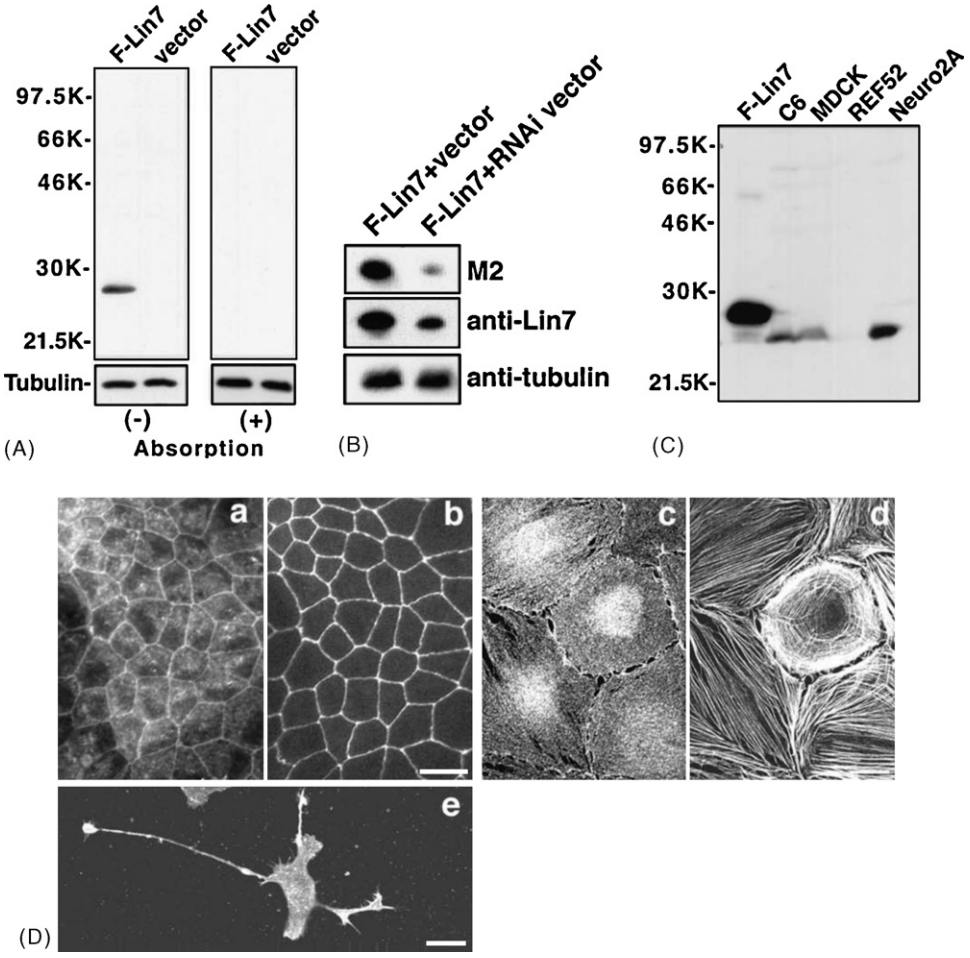


Fig. 3. Characterization of anti-Lin-7 antibody. (A) Lysates from COS7 cells (30 μg of proteins) expressing Flag-Lin-7B (left lane of each panel) or Flag-tag (right lane of each panel) were immunoblotted with anti-Lin-7 (upper left panel) or the antibody preabsorbed by the antigen (upper right panel). The blot membranes were re-probed with anti-tubulin antibody for loading control (lower panels). (B) COS7 cells were double-transfected with pRK5-Flag-Lin-7B and pcDNA6.2-GW/EmGFP-miR-Lin-7 or the vacant vector. The cell lysates (30 μg of proteins) were immunoblotted with M2 (top panel) or anti-Lin-7 (middle panel). A loading control blot with anti-tubulin antibody was also shown (lower panel). (C) Detection of endogenous Lin-7 proteins in cultured cell lines. Lysates (30 μg of proteins) of COS7 expressing Flag-Lin-7B, C6, MDCK, REF52 and Neuro2A cells were subjected to SDS-PAGE (15% gel) and Western blotting with anti-Lin-7. Molecular size markers are at left. (D) Localization of Lin-7 in MDCK (a and b), REF52 (c and d) and Neuro2A (e) cells. MDCK and REF52 cells were double-stained using anti-Lin-7 (a and c) with anti-ZO-1 antibody (b) or rhodamine-phalloidin (d). Neuro2A cells were stained for Lin-7 (e). Bars, 20 μm (b and d) and 10 μm (e).

similar to that of Lin-7, it should be noted that Rhotekin was also localized in the nucleus (Fig. 5D–F).

### 3.4. Interaction of Rhotekin with Lin-7 in the rat brain

Immunocomplex formation of Rhotekin and Lin-7 expressed in COS7 cells strongly suggests their physiological interaction in tissues. We thus tested if endogenous Rhotekin and Lin-7 associate with each other in the brain. We first confirmed that anti-Rhotekin immunoprecipitates Myc-Rhotekin with Flag-Lin-7B expressed in COS7 cells (Fig. 6A and B). When endogenous Rhotekin was immunoprecipitated from extracts of adult rat brains with anti-Rhotekin, Lin-7 was detected in the precipitate (Fig. 6C). On the other hand, Rhotekin per se was hard to detect since the molecular mass of Rhotekin is similar to that of IgG heavy chain (data not shown). These results strongly suggest that Rhotekin interacts with Lin-7 in neuronal tissues. Taken together with the observation that Rhotekin is also concentrated in synapses, we concluded that Lin-7 co-localizes and interacts with Rhotekin in the synapse. These results imply possible cooperative role(s) of Rhotekin and Lin-7 in the regulation of synapse function(s) and/or morphology.

## 4. Discussion

The proper targeting of proteins at the apical or basolateral surface of epithelial cells is crucial for cellular transport. Similarly, proper targeting of proteins to either axons or dendrites plays an important role in neurotransmission. Many studies have been directed at the molecular basis that controls these targeting decisions.

Rho family proteins are important regulators of neuronal cell functions including axon guidance, dendrite formation and spine morphogenesis (Negishi and Katoh, 2002; Bito, 2003; Govek et al., 2005). Physiological functions of Rho family proteins have been extensively analyzed in cultured

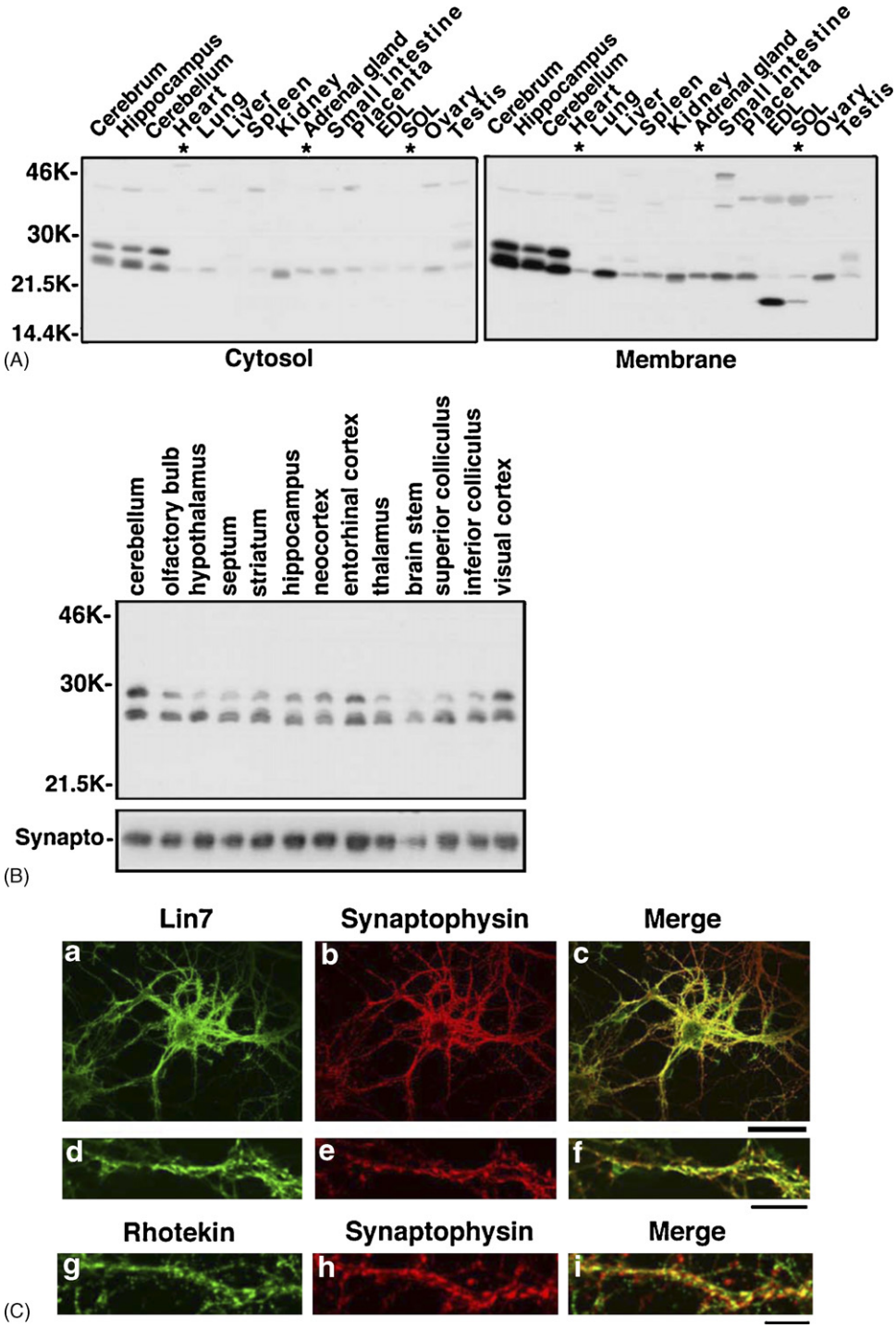


Fig. 4. Detection of Lin-7 in the adult rat tissues and brain. (A) Tissue distribution of Lin-7 in rat tissues. Cytosolic (20 μg of proteins) and membrane (30 μg of proteins) fractions from tissues were separated by SDS-PAGE (15%) and then subjected to Western blotting using anti-Lin-7. (B) Regional expression of Lin-7 in the brain. Various regions were dissected and whole extracts were prepared. Each extracts containing 20 μg of proteins were subjected to SDS-PAGE (15% gel) followed by Western blotting using anti-Lin-7 (upper panel) and anti-synaptophysin (lower panel). Molecular size markers were at left. (C) Rat hippocampal neurons cultured for 14 days were double-stained for Lin-7 (a and d) or Rhotekin (g) with synaptophysin (b, e and h). The merged images are shown (c, f and i). Bars, 10 μm (c) and 2 μm (f and i).

hippocampal neurons. With regard to Rho, the signaling is thought to play essential roles at least by regulating collapsing response mediator protein-2 (CRMP-2) (Fukata et al., 2002). CRMP-2 is a prominent substrate of a Rho effector, Rho-kinase, and has been shown to promote axon formation by regulating the microtubule cytoskeleton. In contrast, physiological functions of Rhotekin in neurons and other tissues are almost unknown.

We here demonstrated that Rhotekin interacts with Lin-7B in vitro and in cells, and forms complex in the brain tissues.

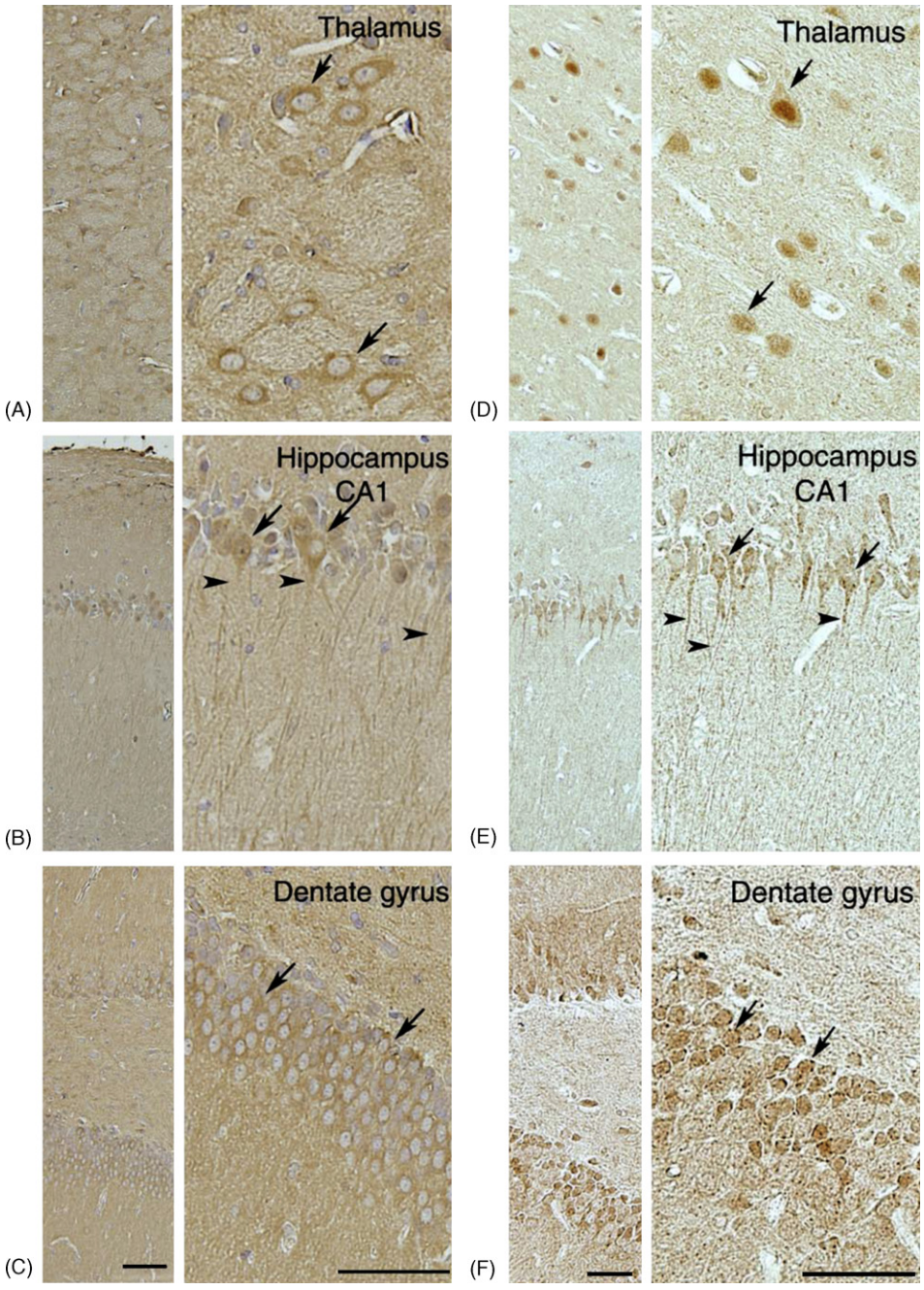


Fig. 5. Immunohistochemical analyses of Lin-7 in the adult rat brain. Immunostaining of Lin-7 (A–C) or Rhotekin (D–F) in thalamus (A and D), hippocampus CA1 area (B and E) and dentate gyrus (C and F). Higher magnified images in each region were also shown at right. Arrows, neurons; arrowheads, axon or dendrite. Sections (A–C) were counterstained with hematoxylin. Note that counterstaining was not done for (D–F) to show the Rhotekin localization at the nucleus. Bars, 50 μm.

The result that active Rho facilitates Lin-7-Rhotekin interaction suggests that their interaction is regulated by Rho signals and Rho/Rhotekin signal may affect the function of Lin-7. This interaction raised a possibility on a novel function of Rhotekin other than control of gene transcription and septin filament organization so far described (Reynaud et al., 2000; Liu et al., 2004; Ito et al., 2005). Interaction of Rhotekin with Lin-7 implies the involvement of Rhotekin in the functions and/or regulation of Lin-2/Lin-7/Lin-10 complex in synapses (Borg et al., 1998; Butz et al., 1998), although further intensive analyses are required for elucidating this issue.

Rhotekin interacts with PIST which is thought to participate in the trafficking of protein in synapses (Cuadra et al., 2004; Ito et al., 2006). Binding of Rhotekin with the PDZ domain of PIST and Lin-7 suggests possible competition of PIST and Lin-7 for the C-terminal PDZ-binding motif of Rhotekin. It is tempting to speculate that the balance between PIST-Rhotekin and Lin-7-Rhotekin complexes might determine synapse functions such as neurotransmitter release.

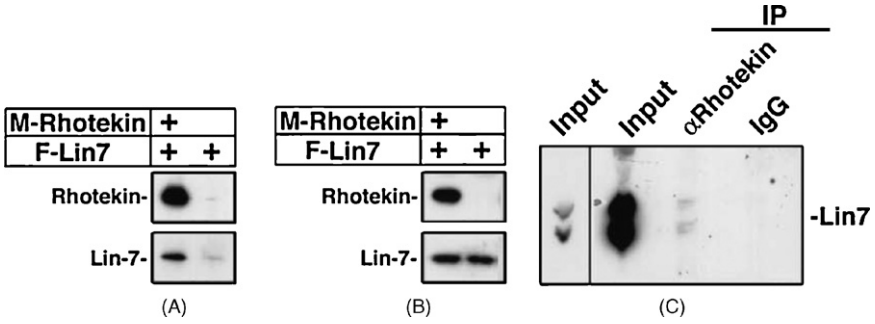


Fig. 6. Immunocomplex formation of endogenous Lin-7 with Rhotekin in the rat brain. (A) Lysates from COS7 cells expressing Flag-Lin-7B with or without Myc-Rhotekin (90% of total protein amounts) were immunoprecipitated with anti-Rhotekin as described in Section 2. Twenty percent of the precipitated materials were subjected to SDS-PAGE (15% gel) followed by Western blotting with a mixture of M2 and 9E10. (B) Expression of each protein in (A) was confirmed by Western blotting of cell lysates (3% of total volume) with a mixture of M2 and 9E10. (C) Rhotekin was immunoprecipitated with anti-Rhotekin from the brain extracts as in (A). Twenty percent of the precipitated materials were subjected to SDS-PAGE (15% gel) followed by Western blotting with anti-Lin-7. The brain extract (input, 1%) was used as a control. Shortly exposed autoradiogram of the input was shown at left.

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