

RanBPM is an L1-interacting protein that regulates L1-mediated mitogen-activated protein kinase activation

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Abstract

A yeast two-hybrid screen using the last 28 amino acids of the cytoplasmic domain of the neural cell adhesion molecule L1 identified RanBPM as an L1-interacting protein. RanBPM associates with L1 *in vivo* and the N-terminal region of RanBPM (N-RanBPM), containing the SPRY domain, is sufficient for the interaction with L1 in a glutathione S-transferase pull-down assay. L1 antibody patching dramatically changes the subcellular localization of N-RanBPM in transfected COS cells.

Overexpression of N-RanBPM in COS cells reduces L1-triggered extracellular signal-regulated kinase 1/2 activation by 50% and overexpression of N-RanBPM in primary neurons inhibits L1-mediated neurite outgrowth and branching. These data suggest that RanBPM is an adaptor protein that links L1 to the extracellular signal-regulated kinase/MAPK pathway.

Keywords: adhesion molecule adaptor, axon extension, Ig superfamily.

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The neural cell adhesion molecule L1 is important for a variety of developmental processes, including neurite outgrowth (Lagenaur and Lemmon 1987), axon fasciculation (Kunz *et al.* 1998), myelination (Haney *et al.* 1999) and migration of neuronal precursors (Lindner *et al.* 1983). Mutations in the human L1 gene cause a number of X-linked disorders, such as X-linked hydrocephalus, agenesis of the corpus callosum and spastic paraplegia type I (Kenwrick *et al.* 1996; Fransen *et al.* 1997; Kamiguchi *et al.* 1998a). Furthermore, L1 knockout mice exhibit several phenotypes similar to human patients with L1 mutations, such as hypoplasia of the corticospinal tract and corpus callosum, hypoplasia of the cerebellar vermis and hydrocephalus (Dahme *et al.* 1997; Cohen *et al.* 1998; Fransen *et al.* 1998).

The extracellular domain of L1, composed of six Ig domains and five fibronectin type III repeats, is capable of binding to a variety of ligands, including L1 itself, other members of the Ig superfamily, integrins, neuropilin and several extracellular matrix components (for review, see Haspel and Grumet 2003). L1 can bind to ligands in *trans* to mediate adhesion or bind to ligands in *cis* to function as coreceptors. There is compelling evidence that the L1 cytoplasmic domain (L1CD) is crucial for L1 function. The L1CD is highly conserved and mutations in it cause Mental Retardation Aphasia Shuffling Gait and Adducted Thumbs (MASA) syndrome (Fransen *et al.* 1997). The L1CD is

phosphorylated by several kinases and phosphorylation appears to regulate L1 function (Wong *et al.* 1996). L1 is known to activate the extracellular signal-regulated kinase (ERK) pathway and it has been suggested that ERK activation is involved in L1-mediated neurite outgrowth and migration (Schaefer *et al.* 1999; Schmid *et al.* 2000). To date, three proteins, ankyrin, adaptor protein-2 (AP-2) and ezrin, have been shown to interact with the L1CD but their relationship to ERK activation is unclear.

The ankyrin-binding site on the L1CD (amino acids 1204–1229) couples L1 to the underlying actin cytoskeleton. The interaction with ankyrin seems to mediate the stationary behavior of L1 and may play a critical role in the regulation

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Abbreviations used: AP-2, Adaptor Protein-2; EGFP, Enhanced Green Fluorescent Protein; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin peptide; HGF, Hepatocyte Growth Factor; IPTG, Isopropyl- β -D-thiogalactopyranosid; L1CD, L1 cytoplasmic domain; LFA-1, lymphocyte function-associated antigen-1; MASA, Mental Retardation Aphasia Shuffling Gait and Adducted Thumbs; MEK, MAPK/ERK kinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

of L1-mediated adhesion and migration (Gil *et al.* 2003). L1 can bind to the clathrin adaptor AP-2 through the YRSL motif and this interaction is critical for clathrin-mediated L1 endocytosis (Kamiguchi *et al.* 1998b). AP-2-mediated L1 endocytosis is critical for L1 recycling at the growth cone (Kamiguchi and Lemmon 2000), sorting of L1 to axons in dorsal root ganglion neurons and L1 transcytosis in hippocampal neurons (Kamiguchi and Lemmon 1998; Wisco *et al.* 2003). The L1CD also binds to ezrin, a member of the ezrin, radixin and moesin family of membrane-cytoskeleton-linking proteins (Dickson *et al.* 2002), through the YRSL motif and the juxtamembrane region (Cheng *et al.* 2005). This interaction provides a link between L1 and the actin cytoskeleton and plays a critical role in the regulation of neurite branching (Cheng *et al.* 2005).

As no interactor with the L1CD has a clear relationship to ERK activation, we sought to identify additional L1 binding proteins by performing a yeast two-hybrid screen. We chose the last 28 amino acids of the L1CD as bait because we have previously shown that this region is phosphorylated (Schaefer *et al.* 1999) but no protein interactions have been reported for this region. We identified RanBPM as an L1-interacting protein. RanBPM was originally cloned because it interacts with RAN, a Ras-like small GTPase, that functions as a carrier in nuclear-cytoplasmic exchange (Nakamura *et al.* 1998). Subsequently, a number of studies have identified RanBPM as a binding partner with several unrelated proteins, such as the hepatocyte growth factor (HGF) receptor Met (Wang *et al.* 2004), integrin lymphocyte function-associated antigen-1 (LFA-1) (Denti *et al.* 2004) and serine/threonine kinase Mirk/Dyrk (Zou *et al.* 2003). RanBPM has also been shown to associate directly with the guanine nucleotide exchange factor Sos and to stimulate Ras/ERK (Wang *et al.* 2002). It also regulates the transcriptional activity downstream of several receptors (Rao *et al.* 2002; Wang *et al.* 2002; Denti *et al.* 2004).

We have demonstrated that L1 and RanBPM interact both *in vitro* and *in vivo*. The N-terminus of RanBPM was sufficient for the interaction with L1. In transfected cells, L1 and RanBPM colocalized in the plasma membrane and antibody-induced L1 patching caused redistribution of RanBPM with substantial colocalization with L1. Overexpression of the N-terminal fragment of RanBPM decreased L1-induced ERK activation by twofold in COS cells and partially inhibited L1-mediated neurite outgrowth in cerebellar neurons. These data suggest that RanBPM serves as an adaptor in L1-mediated signaling involved in neurite growth.

Materials and methods

Materials

All cell culture reagents were from Gibco (Carlsbad, CA, USA). The Nucleofector transfection kit was from Amaxa (Cologne, Germany).

All chemicals were from Sigma (St Louis, MO, USA). Glutathione-Sepharose 4B beads, pGEX-4T-1 vector and anti-glutathione *S*-transferase (GST) were from Amersham Biosciences (Piscataway, NJ, USA). Protease inhibitors and anti-HA were purchased from Roche (Indianapolis, NJ, USA). Polyclonal anti-ERK2 (sc-154) and monoclonal anti-phosphorylated ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-human L1 antibody, rabbit anti-rat L1 antibody and monoclonal anti-L1 antibody 74-5H7 were produced in the laboratory (Schaefer *et al.* 2002). The anti-RanBPM antibody (5M) was a kind gift from Dr T. Nishimito (Kyushu University, Japan). The monoclonal anti-RanBPM antibody was a kind gift from Dr E. Bianchi (Institut Pasteur, Paris, France).

Yeast two-hybrid screen

A cDNA encoding the last 28 amino acids of the L1CD was fused to sequences encoding the GAL4-DNA binding domain in the pGBKT7 vector (Clontech, Palo Alto, CA, USA) to make the vector pGBKT7-L1CD28. The bait lacked any intrinsic transcription activation activity or toxicity to the host strain. A pre-transformed adult human brain two-hybrid prey cDNA library in the Y187 yeast strain (Clontech) was screened by mating with AH109 transformed with the bait construct pGBKT7-L1CD-28. Positive clones were selected on SD/-Leu/-Trp/-Ade/-His/15 mM 3-aminotriazole. Plasmid DNAs from positive clones were isolated and used to reverify the interaction. To map the binding region, various combinations of the bait vector pAS2-L1CD or pGBKT7-L1CD28 and the prey vector pACT2-tRanBPM, pACT2-N-terminal region of RanBPM (N-RanBPM) or pACT2-C-terminal region of RanBPM were cotransformed into AH109 and grown on low stringency plates (SD/-Leu/-Trp/-His/5 mM 3-aminotriazole) to test the interaction.

Plasmid construction

The pcDNA3-human L1 vector (Wong *et al.* 1995) and the pAS2-L1CD vector (Kamiguchi *et al.* 1998b) were described previously. The mammalian expression vector pcDNA3-N-RanBPM encodes amino acids 117–354 of RanBPM with an HA tag at the N-terminus. The yeast prey vectors pACT2-N-RanBPM and pACT2-C-terminal region of RanBPM encode amino acids 117–354 and 355–729 of RanBPM, respectively. The GST-tagged bacterial recombinant expression vector pGEX-N-RanBPM was generated by inserting codons 117–354 of RanBPM into pGEX-4T-1.

Glutathione *S*-transferase pull-down assay

pGEX-N-RanBPM was transformed into BL21 cells and induced with 1 mM Isopropyl- β -D-thiogalactopyranosid (IPTG) for 4 h at 37°C. GST fusion proteins were purified using glutathione-Sepharose 4B beads. Equal amounts of GST or GST-N-RanBPM (~100 μ g protein on 100 μ L 50% slurry of beads) were incubated with glutathione-Sepharose 4B beads for 2 h at 4°C and then washed twice with the binding buffer (20 mM HEPES, 50 mM NaCl, 0.1% Tween-20, pH 7.4). Immunoaffinity-purified mouse L1 (50 μ L) (see below) was added to the beads and incubated overnight at 4°C. The beads were washed three times with the binding buffer. Bound proteins were eluted with 30 μ L sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% gel) followed by western blotting with anti-GST (1 : 2000) and anti-rat L1 (1 : 1000).

L1 affinity purification

The procedure was performed as described previously (Schaefer *et al.* 1999) with slight modifications. Briefly, brains from adult mice were homogenized in 50 mM Tris, pH 7.5, 1 mM pervanadate, 0.32 M sucrose and EDTA-free complete protease inhibitors (Roche). The homogenates were separated by ultracentrifugation on a sucrose gradient for 45 min at 58 400 *g*. The plasma membrane layer was collected and then centrifuged for 30 min at 150 000 *g* to pellet the membranes. The plasma membrane pellet was solubilized in 50 mM Tris, pH 7.5, with 1% Triton-X-100 and centrifuged for 45 min at 150 000 *g* to remove the insoluble material. The solubilized membrane fraction was run through the 74-5H7 column overnight at 4°C. Bound proteins were eluted with 0.1 M diethylamine (pH 11.2) and immediately neutralized with Tris (2 M, pH 4). Peak fractions were dialysed with 50 mM Tris, pH 7.5, overnight at 4°C and then resolved on SDS-PAGE followed by silver staining to verify the band patterns. To test the interaction between L1 and RanBPM, 1 µg of immunoaffinity-purified mouse L1 was resolved on 7.5% SDS-PAGE followed by western blotting with rabbit anti-RanBPM (1 : 2000) and mouse anti-RanBPM (1 : 1000).

Western blot analysis

Samples were mixed with SDS sample buffer with 2-mercaptoethanol, boiled for 5 min and then separated by SDS-PAGE. The proteins were transferred to Immobilon-P membrane for immunoblotting. For chemiluminescent western blots, membranes were blocked with 10% bovine serum albumin in Tris-buffered saline. Horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and SuperSignal West Pico chemiluminescence kit (Pierce, Rockford, IL, USA) were used for detection. For western blots with the Odyssey Infrared Fluorescence Imager (LI-COR, Lincoln, NE, USA), the membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline. IRDye 800- (Rockland, Gilbertsville, PA, USA) or Alexa 680- (Molecular Probes, Eugene, OR, USA) coupled secondary antibodies were used.

Cell culture and transfection

COS-7 cells were grown in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and GlutaMAX. Transient transfection was performed using the Nucleofector electroporation device according to the manufacturer's protocol (program A24). Cells were grown for 48 h after transfection.

Immunofluorescence

For immunofluorescence staining, transfected COS-7 cells were grown on glass coverslips for 48 h. Cells were fixed with 4% paraformaldehyde and permeabilized in phosphate-buffered saline with 0.03% Triton-X-100. Cells were incubated with the corresponding primary antibodies (1 : 1000 rabbit anti-hL1 for the pcDNA3-human L1 vector or 2 µg/mL anti-HA for the pcDNA3-N-RanBPM) followed by incubation with secondary antibodies (1 : 200 Alexa 488 anti-mouse IgG and 1 : 200 Texas Red-X anti-rabbit IgG). In the experiments involving antibody patching, COS-7 cells were cotransfected with pcDNA3-human L1 and pcDNA3-N-RanBPM/pFLAG-RanBPM and grown for 48 h after transfection. Live cells were incubated with 1 : 100 heat-inactivated rabbit anti-human L1 antibody for 10 min at 37°C. Cells were then fixed, permeabilized and stained with anti-HA antibodies, followed by incubation with

Alexa 488 goat anti-mouse IgG and Texas Red-X anti-rabbit IgG. Images were acquired with a confocal laser microscope (LSM 510; Zeiss, Oberkochen, Germany) using an Argon and HeNe laser and a 100× Plan Neofluor (N.A. 1.3) oil objective.

Extracellular signal-regulated kinase activation

COS-7 cells were transfected with the pcDNA3-human L1 vector alone or cotransfected with the pcDNA3-human L1 vector and the pcDNA3-HA-N-RanBPM vector. Transfected cells were plated at a density of 1×10^6 cells/35-mm dish. At 24 h after transfection, cells were serum starved for 16–20 h. The cells were then treated with heat-inactivated rabbit anti-human L1 antibody (1 : 100) for various periods. After the treatment, cells were lysed in 100 µL lysis buffer/35-mm dish (50 mM Tris, 150 mM NaCl, pH 7.2, 1% Triton X-100 and EDTA-free Complete protease inhibitors). Lysates were collected and centrifuged at 16 000 *g* for 5 min to remove the insoluble material. The supernatant fluid was collected and mixed with sample buffer. Samples were boiled for 5 min and resolved on SDS-PAGE (10% gels) followed by western blotting. To detect ERK activation, two-color western blot was performed with rabbit anti-total ERK2 (1 : 500) and mouse anti-phosphorylated ERK (1 : 500) followed by incubation with Alexa 680 goat anti-mouse IgG (1 : 5000) and IRDye goat anti-rabbit IgG (1 : 5000). Another two-color western blot was performed with rabbit anti-human L1 (1 : 5000) and mouse anti-HA (1 µg/mL) followed by incubation with Alexa 680 goat anti-mouse IgG (1 : 5000) and IRDye goat anti-rabbit IgG (1 : 5000). To perform the quantitative analysis, the integrated intensity for the ERK1/2 band on the original scan was quantified with the Odyssey software (LI-COR). ERK activation was calculated by dividing the integrated intensity of the band in the treated samples by the integrated intensity of the band in the untreated samples. Similar results were obtained in triplicate experiments.

Neurite outgrowth analysis

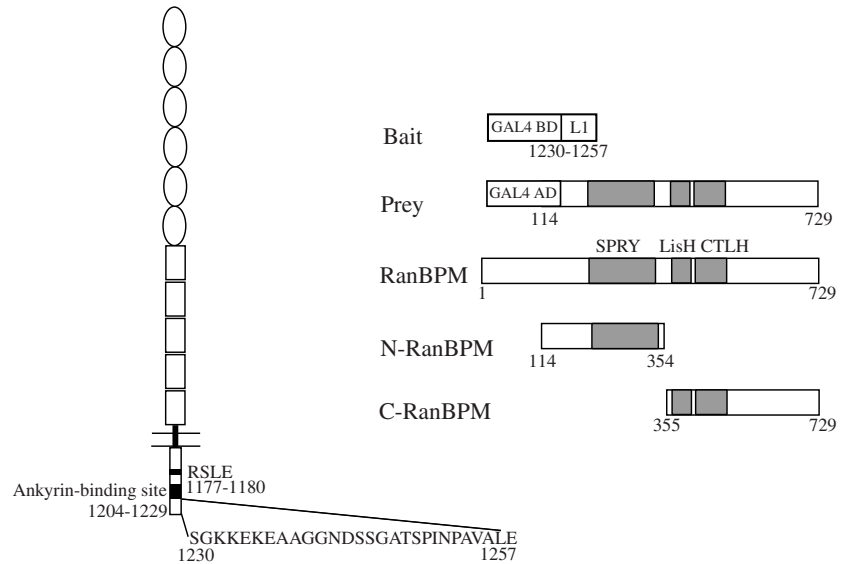
The preparation of cerebellar neuron cultures, L1 substrates and electroporation was described previously (Cheng and Lemmon 2004). Briefly, post-natal day 8 mouse cerebella were dissected and dissociated into single cells by trypsin and deoxyribonuclease treatment. Cells were transfected with the Nucleofector electroporation device (program G13) and plated onto L1-coated coverslips. After 48 h, cells were fixed, permeabilized and incubated with anti-HA antibodies to detect N-RanBPM-expressing neurons. Images were acquired with a Spot CCD camera RT slider (Diagnostic Instruments, Sterling Heights, MI, USA) coupled to a DLMB microscope (Leica, Nussloch, Germany) with a 20× objective. Images were analysed with NeuroLucida (MicroBrightField Inc., Williston, VT, USA) and figures prepared with Photoshop 7 (Adobe, San Jose, CA, USA). Quantification of neurite outgrowth was performed as described by Cheng and Lemmon (2004).

Results

Identification of RanBPM as a L1-interacting protein by yeast two-hybrid screening

We performed a yeast two-hybrid screen on a pre-transfected adult human brain cDNA library using the last 28

Fig. 1 Identification of RanBPM as an L1-interacting protein by yeast two-hybrid screen. The structures of L1, bait protein with last 28 amino acids of the L1 cytoplasmic domain, longest prey clone isolated in the screen, complete RanBPM protein, N-terminal region of RanBPM (N-RanBPM) and C-terminal region of RanBPM (C-RanBPM) construct are shown.



amino acids of the L1CD as bait. Twenty-five positive clones were identified after screening 4×10^7 clones. DNA sequencing revealed that seven of the positive clones encoded a partial sequence of RanBPM (GeneBank Accession no. AB055311). The longest sequence of the seven library clones was ~ 2.8 kb in length and encoded amino acids 117–729 of RanBPM (Fig. 1). Independent cotransformation of the bait construct pGBKT7-L1CD28 or pAS2-L1CD (complete L1CD fused with the GAL4 activation domain) and the candidate clone pACT2-RanBPM into yeast further confirmed the interaction. RanBPM interacted with both the last 28 amino acids of the L1CD and the complete L1CD (Fig. 2a). The selectivity of this interaction was demonstrated by lack of interaction between the last 28 amino acids of the L1CD and SV40 large T antigen (Fig. 2a).

N-terminal region of RanBPM containing the SPRY domain is sufficient for the interaction with L1

RanBPM contains a SPRY domain (dual-specificity kinase *splA* and ryanodine receptor domain; amino acids 212–333), a LisH domain (Lissencephaly type-1-like homology motif, amino acids 364–397) and a CTLH domain (C-terminal to LisH motif, amino acids 403–459). The function of the SPRY domain is still unclear but several studies have indicated that it is a protein–protein interaction domain (Rao *et al.* 2002; Wang *et al.* 2004). We speculated that the SPRY domain might therefore be involved in the interaction with L1. To test our hypothesis, two prey vectors were constructed. pACT2-N-RanBPM encodes amino acids 117–334 of RanBPM, containing the complete SPRY domain. pACT2-C-terminal region of RanBPM encodes amino acids 335–729 of RanBPM, containing the LisH, CTLH and the remaining C-terminal part. In the yeast two-hybrid assay, N-RanBPM interacted with both the last 28 amino acids of the L1CD and

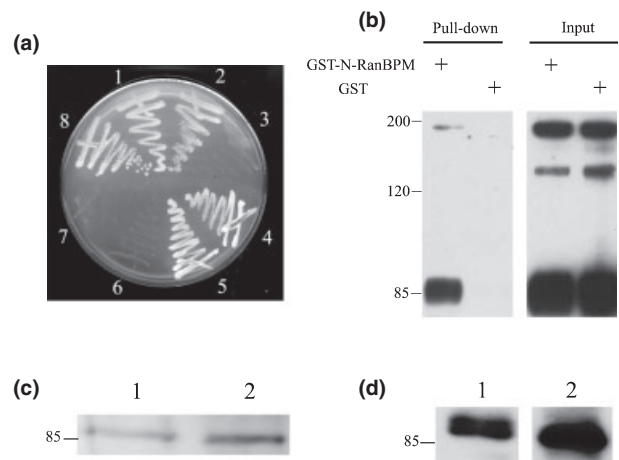


Fig. 2 RanBPM interacts with L1 *in vitro* and *in vivo*. (a) Yeast AH109 cells were cotransformed and selected on a SD/-Trp/-Leu/-His/5 mm 3-aminotriazole plate. (1–3) AH109 yeast strain was cotransformed with pGBKT7-L1 cytoplasmic domain (L1CD)28 and the following GAL4 prey vectors: (1) pACT2-RanBPM; (2) pACT2-N-terminal region of RanBPM (N-RanBPM) and (3) pACT2-C-terminal region of RanBPM (C-RanBPM). (4–6) AH109 strain was cotransformed with pAS2-L1CD and the following GAL4 prey vectors: (4) pACT2-RanBPM; (5) pACT2-N-RanBPM and (6) pACT2-C-RanBPM. (7) Cotransformation of pGBKT7-L1CD28 and large T antigen-AD as negative control. (8) Cotransformation of p53-DBD and large T antigen-AD as positive control. (b) Glutathione *S*-transferase (GST)-N-RanBPM binds to purified L1 in GST pull-down assay. GST or GST-N-RanBPM was immobilized on beads and incubated with purified L1. L1 and an L1 fragment (85 kDa) containing the L1CD were detected on beads immobilized with GST-N-RanBPM but not on GST alone. (c and d) RanBPM was detected in L1 purified from brain. (1) Brain lysates as a positive control; (2) purified L1 from mouse brain. (c) Mouse anti-RanBPM; (d) rabbit anti-RanBPM.

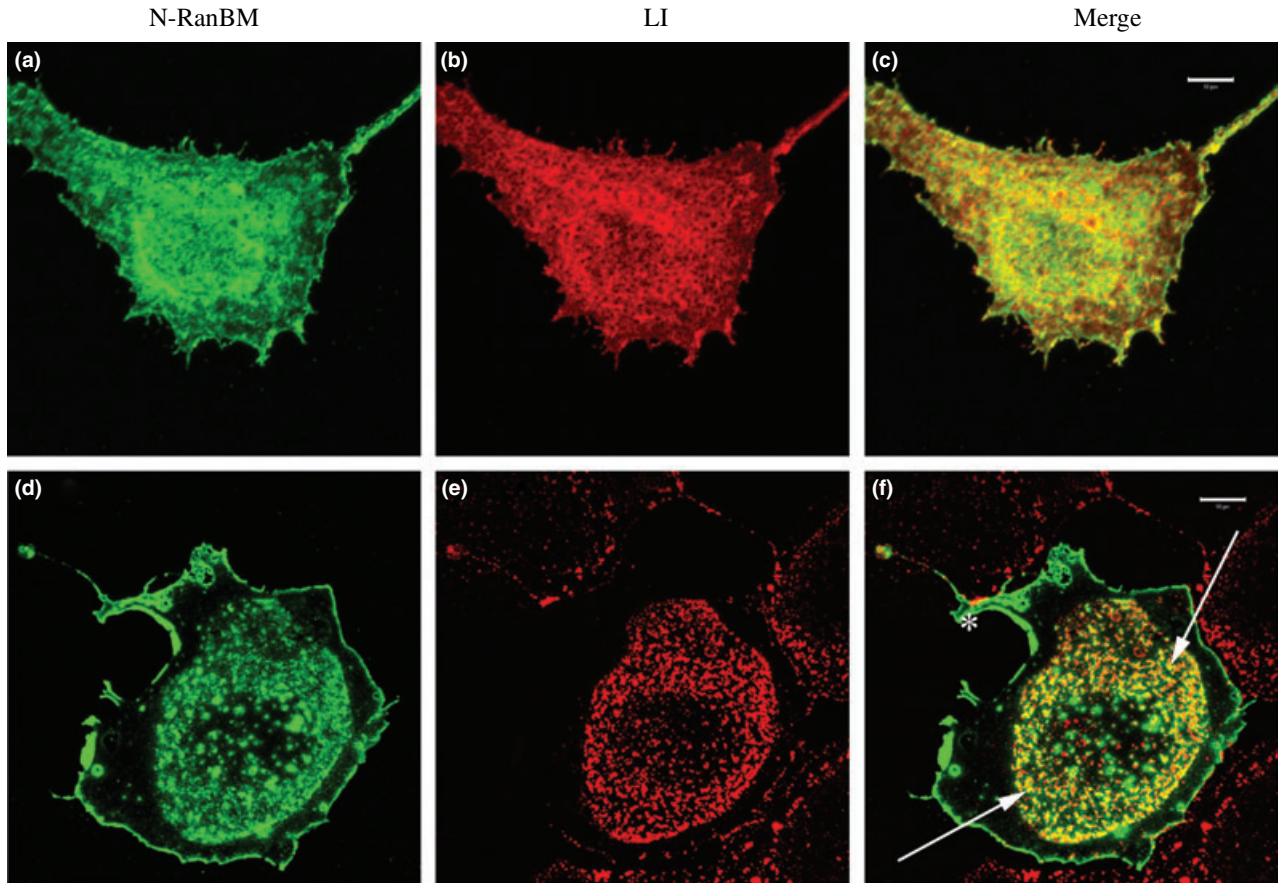


Fig. 3 Colocalization of L1 and N-terminal region of RanBPM (N-RanBPM) after antibody-induced patching of L1. These confocal images represent Z-stacks through the cells. (a–c) COS-7 cells cotransfected with HA-tagged N-RanBPM (a) and L1 (b). Cells were fixed and stained without patching. (c) N-RanBPM (green) showed extensive colocalization with L1 (red). Bar, 10 μ m. (d–f) COS-7 cells cotransfected with HA-tagged N-RanBPM (d) and L1 (e). In this field

only one cell expressed N-RanBPM while adjacent cells expressed some L1. Cells were patched with Ranti-L1 for 10 min before fixation. L1 (red) exhibited a patchy distribution in the center of the cell and N-RanBPM (green) was partially colocalized with L1 patches indicated with arrows. Some RanBPM remained at the perimeter of the cell (asterisk) and is not associated with L1. Bar, 10 μ m.

the complete L1CD, while C-terminal region of RanBPM interacted with neither (Fig. 2a). These results suggest that sequences in N-RanBPM are critical for the interaction with L1. To test this interaction using native L1, a GST fusion protein with the N-RanBPM was purified, immobilized on glutathione beads and used in a GST pull-down assay. Immunoaffinity-purified L1 from mouse brain bound to GST-N-RanBPM but not to GST alone (Fig. 2b). Due to protease cleavage, the L1 preparation contained full-length L1 (~200 kDa), an amino-terminal extracellular fragment (~135 kDa) and a C-terminal fragment containing the L1CD (~85 kDa) (Grumet *et al.* 1984). Full-length L1 and the 85-kDa C-terminal bound to GST-N-RanBPM but the 135-kDa extracellular fragment did not, consistent with the notion that GST-N-RanBPM binds to the L1CD. Results from both the yeast two-hybrid and the GST pull-down assays suggest that N-RanBPM, containing the SPRY domain, is sufficient for the interaction with L1.

RanBPM interacts with L1 *in vivo*

To investigate whether L1 and RanBPM interact *in vivo*, L1 and L1-associated proteins were purified from mouse brain and detected with two different antibodies against RanBPM, a rabbit antibody (Nishitani *et al.* 2001) and a mouse monoclonal antibody (Denti *et al.* 2004). Both antibodies detected a 90–100-kDa band in immunoaffinity-purified L1 samples (Fig. 2c, lane 2 and Fig. 2d, lane 2) in the range of the reported RanBPM molecular weight (Nakamura *et al.* 1998). As a positive control, the same antibody also detected a band of similar molecular weight in the brain lysate samples (Fig. 2c, lane 1 and Fig. 2d, lane 1). This strongly suggests that RanBPM associates with L1 *in vivo*.

Colocalization of L1 and N-terminal region of RanBPM after antibody-induced patching of L1

The biochemical studies above argue that L1 interacts with RanBPM. Colocalization of L1 and RanBPM in cells would

lend support to this hypothesis. Preliminary studies indicated that HA-tagged, full-length RanBPM in pcDNA3 gave poor expression in COS-7 cells but that HA-tagged N-RanBPM was expressed at acceptable levels. These studies also indicated that the N-RanBPM was concentrated along the plasma membrane (Fig. 3a) and that L1 and N-RanBPM showed good colocalization. Colocalization of two membrane-associated molecules in the plasma membrane does not prove that they interact so we hypothesized that altering the distribution of L1 would also redistribute RanBPM if they interact. Therefore, antibody-induced patching of L1 was performed on COS-7 cells cotransfected with L1 and HA-tagged N-RanBPM. We performed L1 patching assays with a short antibody incubation time (10 min at 37°C and no secondary antibody), which is sufficient to induce L1 patching as well as some internalization (Long *et al.* 2001). Confocal microscopic inspection revealed that most of the cells exhibited a patchy distribution of L1 (Fig. 3e) and a redistribution of N-RanBPM into the patches while some RanBPM remained at the perimeter of the cells. There is extensive colocalization between the L1 and N-RanBPM clusters (Fig. 3f), suggesting that L1 interacts with N-RanBPM and L1 can influence the distribution of N-RanBPM.

N-terminal region of RanBPM inhibits L1-mediated MAPK activation

Previous studies demonstrated that RanBPM directly interacts with Sos to activate Ras and induce ERK phosphorylation (Wang *et al.* 2002). Also, overexpression of full-length RanBPM led to the constitutive activation of the Ras-ERK pathway (Wang *et al.* 2002). As L1 cross-linking is known to activate ERK (Schaefer *et al.* 1999; Schmid *et al.* 2000; Loers *et al.* 2005), we hypothesized that RanBPM might function as an adaptor to mediate L1-induced ERK activation. To test this hypothesis, we performed L1 cross-linking with polyclonal anti-human L1 antibodies on both COS-7 cells transfected with L1 alone and COS-7 cells cotransfected with L1 and N-RanBPM. In COS-7 cells transfected with L1 alone, L1 cross-linking triggered a strong activation of ERK, as previously described in NIH 3T3 cells (Schaefer *et al.* 1999). Maximum activation was observed within 10 min of stimulation (4.55-fold increase in ERK activity, $n = 3$, SD, 0.28) and the activation went down to the basal level after 30 min of stimulation (Fig. 4). In cells cotransfected with L1 and N-RanBPM, antibody cross-linking also triggered the activation of ERK after 10 min of treatment but the activation was weaker compared with cells transfected with L1 alone (2.00-fold increase in ERK activity, $n = 3$, SD, 0.08). This was not due to a difference in L1 expression level, as shown by the blot probed with anti-L1 (Fig. 4). Quantitative analysis demonstrated that N-RanBPM reduced the L1-induced ERK activation by twofold. This suggests that RanBPM is involved in L1-induced ERK activation.

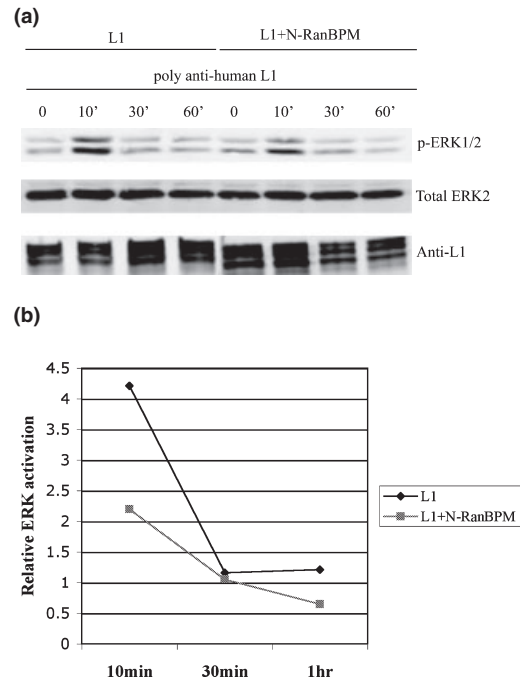


Fig. 4 N-terminal region of RanBPM (N-RanBPM) inhibits extracellular signal-regulated kinase (ERK) activation induced by L1 cross-linking. (a) Serum-starved COS-7 cells transfected with L1 alone or cotransfected with L1 + N-RanBPM were treated with polyclonal anti-L1 for various time periods. ERK activation was detected with anti-phosphorylated ERK. The blot with total ERK2 was to ensure equal loading and the blot with anti-L1 was to ensure equal L1 expression level between samples. (b) Densitometric analysis of the relative ERK activation change induced by antibody cross-linking. A summary of three experiments is shown. Error bars, SD.

N-terminal region of RanBPM reduces L1-mediated neurite outgrowth

As L1-mediated neurite outgrowth involves MAPK activation (Schmid *et al.* 2000), we speculated that RanBPM might regulate L1-mediated neurite outgrowth through the MAPK pathway. We transfected the N-RanBPM vector pcDNA3-N-RanBPM into wild-type cerebellar neurons and grew transfected cells on an L1 substrate (Fig. 5b). As a positive control, we also transfected Enhanced Green Fluorescent Protein (EGFP) vector into neurons (Fig. 5a). EGFP-transfected neurons had an average neurite length of 240 μm after 48 h. In comparison, the N-RanBPM-expressing neurons had an average longest neurite length of 206 μm , a 14% decrease. This decrease was significant at the $p < 0.02$ level and is representative of three experiments (Fig. 5d). On the neurite length distribution curve (Fig. 5c), there was a significant shortening of neurites in the N-RanBPM-expressing neurons compared with EGFP-expressing neurons. Interestingly, when we examined total neurite length, as opposed to single longest neurite, the effect appeared larger with a reduction from 366 to

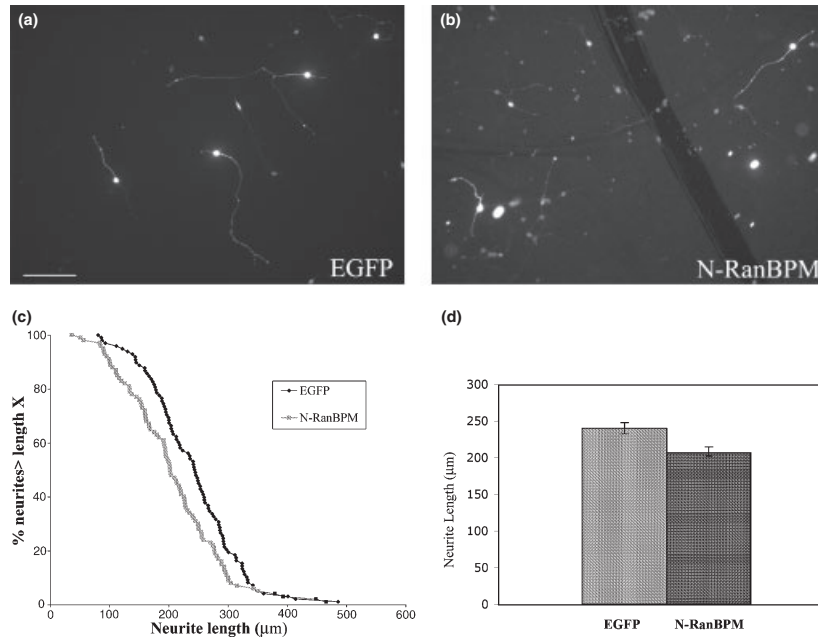


Fig. 5 N-terminal region of RanBPM (N-RanBPM) inhibits L1-mediated neurite outgrowth. (a and b) Cerebellar neurons transfected with EGFP (a) or N-RanBPM (b) were grown on L1 substrate. Cultures were fixed and stained after 48 h. Bar, 100 μm . (c) The distribution

curve of neurite length. The percentage of neurons with neurites longer than any given length was plotted against neurite length. (d) Bar graph showing mean neurite length \pm SEM for neurons expressing EGFP or N-RanBPM.

300 μm , a 18% reduction that was significant at the $p < 0.0002$ level. This is entirely accounted for by a reduction in secondary branching. While the number of primary neurites in N-RanBPM-treated cells was virtually identical to that in EGFP-transfected neurons (2.14 ± 0.06 vs. 2.13 ± 0.04 , respectively, mean \pm SEM), the number of secondary branches was reduced by 53% (0.91 ± 0.15 vs. 1.93 ± 0.21 , $p < 0.002$). This result supports the idea that N-RanBPM functions as a dominant-negative adaptor to inhibit the L1-induced activation of the ERK pathway and thus reduces L1-mediated neurite outgrowth, possibly by having its most significant effect on branching.

Discussion

Using yeast two-hybrid screening, we have identified RanBPM as an L1-binding protein. RanBPM interacts with L1 *in vitro* and *in vivo*. The N-terminal region of RanBPM, containing the SPRY domain, is sufficient for the interaction with L1. We also demonstrate that N-RanBPM can inhibit L1-induced MAPK activation and L1-mediated neurite outgrowth, suggesting that RanBPM is an adaptor protein that participates in L1-induced MAPK activation.

Previous studies have demonstrated that the RanBPM SPRY domain is sufficient for interaction with Met and the serine/threonine kinase Mirk/Dyrk1B (Wang *et al.* 2002; Zou *et al.* 2003). Our study also demonstrates that the N-RanBPM containing the complete SPRY domain is

sufficient for the interaction with L1 in the GST pull-down and yeast two-hybrid assays. Denti *et al.* (2004) have demonstrated that RanBPM can be phosphorylated both constitutively and in response to stress. Osmotic shock induced a shift towards hyperphosphorylated forms of RanBPM. Pre-treatment of cells with p38 kinase inhibitor blocks this phosphorylation, suggesting that the p38 kinase may phosphorylate RanBPM in response to stress. The phosphorylation site predicted by Scansite (<http://www.scansite.mit.edu>) identifies a p38 kinase phosphorylation site at Thr320, inside the SPRY domain; this site is conserved across species, including human, mouse, *Xenopus*, *Caenorhabditis elegans* and yeast (Wang *et al.* 2002). Therefore, the p38 kinase phosphorylation site inside the SPRY domain may be physiologically relevant and may regulate protein interactions with RanBPM in response to stress.

Although RanBPM was first identified as a centrosomal protein, Denti *et al.* (2004) have demonstrated, using a monoclonal antibody against RanBPM, that at least a fraction of RanBPM associates with the plasma membrane. Similarly, we found that HA-tagged N-RanBPM can be localized to the plasma membrane, based on confocal microscopy. The plasma membrane localization of RanBPM, together with RanBPM's role in the ERK pathway and its interaction with several transmembrane proteins (including integrin LFA-1, HGF receptor Met and L1), suggest that RanBPM can function as an adaptor protein for transmembrane proteins on the cell surface.

L1 is known to associate with components of the ERK/MAPK pathway *in vivo* (Schaefer *et al.* 1999). In L1 immunoprecipitates from rat brain, Raf-1, ERK2 (Schaefer *et al.* 1999) and MAPK/ERK Kinase (MEK)-1 (VL, unpublished data) can be detected. Furthermore, L1 cross-linking can activate ERK (Schaefer *et al.* 1999; Schmid *et al.* 2000). However, it is not clear how L1 activates the ERK pathway after L1 cross-linking. The direct association of RanBPM with L1 and the ability of RanBPM to recruit Sos and stimulate Ras activation (Wang *et al.* 2002) suggest that RanBPM may be the adaptor protein for L1 to activate MAPK. We demonstrate that the ERK1/2 activation in COS cells coexpressing L1 and N-RanBPM is weaker than that in COS cells expressing only L1. Quantitative analysis shows a twofold difference in ERK activation. This suggests that N-RanBPM acts as a dominant-negative RanBPM to block L1-induced MAPK activation. The underlying mechanism is still unclear. It is possible that N-RanBPM can compete with endogenous RanBPM to bind L1 but fails to recruit Sos and thus cannot activate ERK efficiently. However, Maness and associates have argued that ras is not involved in the L1 activation of ERK (Schmid *et al.* 2000) based on pharmacological studies. It is not clear whether this is due to a difference in cell types or whether RanBPM can influence erk independent of Sos and ras.

Overexpressing N-RanBPM reduces neurite growth to a small but statistically significant degree (14%). One could question whether this decrease is biologically relevant. However, we have recently demonstrated that deletion of 110 of 114 amino acids of the L1CD decreases neurite length on L1 substrates by 17% (Cheng *et al.* 2005), which is remarkable given the very high conservation of the L1CD. It is, in fact, similar to the effect of overexpressing N-RanBPM on neurite length. This suggests that loss of interaction with RanBPM accounts for most of the effects of the L1CD on neurite length and RanBPM is a regulator of neurite length. Other studies have shown that the MAPK pathway is required for L1-mediated neurite outgrowth, as the MEK inhibitors PD98059 and U0126 can block L1-stimulated neurite outgrowth (Schmid *et al.* 2000; Watanabe *et al.* 2004; Loers *et al.* 2005). In our study, N-RanBPM overexpression decreases neurite length by 14%, presumably by decreasing ERK activation. The less potent effect of N-RanBPM overexpression, compared with the MEK inhibitors, is probably because the N-RanBPM inhibits L1-induced ERK activation by 50% but the MEK inhibitors can block ERK activation completely. Nonetheless, both studies suggest that L1-mediated neurite outgrowth requires activation of the MAPK pathway and RanBPM is involved in L1-mediated neurite outgrowth through its role in MAPK signaling. A more refined analysis revealed that N-RanBPM had a slightly larger effect on overall neurite growth (an 18% reduction in total neurite length) that is probably explained by a large (50%) reduction in secondary branch formation. In

unpublished time-lapse studies we have found that secondary branching is not common in our cerebellar cultures until about 48 h have elapsed. Earlier studies on erk and src inhibitors of L1-mediated neurite growth (Schmid *et al.* 2000; Watanabe *et al.* 2004; Loers *et al.* 2005) have used conditions (relatively short culture times, 24 h or less) and analysis methods (total neurite growth or percent cells with neurites) that would not have uncovered a role for erk and src in neurite branching. Clearly this needs to be investigated further.

RanBPM associates with the plasma membrane and can bind to numerous transmembrane proteins, including the integrin LFA-1 and the HGF receptor Met as well as the neural cell adhesion molecule L1. The emerging view is that RanBPM may serve as a scaffolding protein to regulate signal transduction. Furthermore, it has been suggested that L1 can interact with integrins in *cis* (Silletti *et al.* 2000). The ability of RanBPM to interact with both L1 and integrins suggests that RanBPM may play a role in the 'cross-talk' between L1 and $\beta 1$ integrin. The ubiquitous expression pattern of RanBPM and its interaction with multiple membrane receptors or cell adhesion molecules place RanBPM in an ideal position to integrate different signaling pathways.

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