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Alsin Is a Rab5 and Rac1 Guanine Nucleotide Exchange Factor*

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***ALS2* is the gene mutated in a recessive juvenile form of amyotrophic lateral sclerosis (*ALS2*). *ALS2* encodes a large protein termed alsin, which contains a number of predicted cell signaling and protein trafficking sequence motifs. To gain insight into the overall function of alsin and to begin to evaluate its role in motor neuron maintenance, we examined the subcellular localization of alsin and the biochemical activities associated with its individual subdomains. We found that the Vps9p domain of alsin has Rab5 guanine nucleotide exchange activity. In addition, alsin interacted specifically with and acted as a guanine nucleotide exchange factor for Rac1. Immunofluorescence and fractionation experiments in both fibroblasts and neurons revealed that alsin is a cytosolic protein, with a significant portion associated with small, punctate membrane structures. Many of these membrane structures also contained Rab5 or Rac1. Upon overexpression of full-length alsin, the overexpressed material was largely cytosolic, indicating that the association with membrane structures could be saturated. We also found that alsin was present in membrane ruffles and lamellipodia. These data suggest that alsin is involved in membrane transport events, potentially linking endocytic processes and actin cytoskeleton remodeling.**

Amyotrophic lateral sclerosis (ALS)[1] is a heterogeneous neurological disorder characterized by progressive degeneration of motor neurons, usually causing death as a result of respiratory paralysis (1, 2). Although mostly sporadic in nature, a genetic link has been established in 5–10% of ALS cases (3). Chromosomal mapping studies have identified six independent loci associated with the familial forms of ALS (Refs. 4–10; reviewed in Refs. 11 and 12). Molecular genetic analysis identified two genes that, when mutated, lead to ALS. The first discovered was the gene coding for Cu-Zn superoxide dismutase 1 (*SOD1*) (10). Initially, mutations in *SOD1* were thought to result in defective free radical scavenger activity. However, it is now generally accepted that the alterations in *SOD1* that lead to ALS are the result of an unknown, but toxic gain-of-function. The second gene identified is mutated in a juvenile form of ALS, *ALS2* (13, 14). Mutations in this gene lead to a rare recessive form of ALS that presents early in life and progresses much more slowly than the classical form (6, 15). Two small deletions in *ALS2* were originally associated with the disease (13, 14). Each is expected to severely truncate the predicted protein product of *ALS2*. In addition, mutations in *ALS2* have recently been associated with two other neurodegenerative disorders, juvenile-onset primary lateral sclerosis (14) and infantile-onset hereditary spastic paralegia (16–18). Like the original mutations identified, these mutations are predicted to generate prematurely truncated forms of the protein product.

The protein encoded by *ALS2*, alsin, is predicted to contain numerous domains implicating roles in cell signaling, membrane localization, and protein transport events (13, 14, 19). The NH_2-terminal region of alsin consists of five repeats that show sequence homology with RCC1 (for regulator of chromatin condensation 1; see Fig. 1*A*). RCC1 has been shown to function as a guanine nucleotide exchange factor (GEF) for the Ran family of GTPases (20). Although more than 90 proteins that contain one or more RCC1 repeats are present in data bases (21), only RCC1 itself has Ran GEF activity. Alsin also contains centrally located diffuse B-cell lymphoma (Dbl) homology (DH) and pleckstrin homology (PH) domains, a hallmark of guanine nucleotide exchange factors for the Rho GTPase family (22). Rho GTPases have been shown to be involved in numerous signaling events, but their role in the regulation of the actin cytoskeleton is the best characterized (23–25). COOH-terminal to the PH domain are eight copies of a sequence motif called MORN (for membrane occupation and recognition nexus) (26). This is a largely uncharacterized domain that is found in a number of proteins, but its function is unknown. At its COOH terminus, alsin also possesses a Vps9p domain. This domain specifically catalyzes guanine nucleotide exchange on Rab5 and the yeast homologue Vps21p, thereby activating the GTPases (27–29). Activation of Rab5 is essential for protein trafficking through the early stages of the endocytic pathway.

To gain insight into the overall function of alsin, we examined the biochemical activities associated with its individual subdomains and explored the subcellular localization of alsin. We found that alsin catalyzed guanine nucleotide exchange on both Rab5 and Rac1. Endogenous alsin localized to the cytoplasm and punctate structures in neurons and fibroblasts. Overexpression of full-length alsin indicated that association with these punctate structures could be saturated. Moreover, endogenous alsin partially colocalized with both Rab5 and Rac. In addition, we found alsin in actin-positive structures such as lamellipodia and membrane ruffles. Taken together, our re-

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[1] The abbreviations used are: ALS, amyotrophic lateral sclerosis; GEF, guanine nucleotide exchange factor; MBP, maltose-binding protein; GFP, green fluorescent protein; RFP, red fluorescent protein; DH, Dbl homology; PH, pleckstrin homology; GST, glutathione *S*-transferase; PBD, Rac GTPase binding domain of p21-activated kinase family; PAK, p21-activated kinase family; Ni-NTA, nickel-nitrilotriacetic acid; BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactoside; MAP2b, microtubule-associated protein 2b; RLD, RCC1-like domain.

sults suggest a potential role for alsin in membrane trafficking events through its regulation of the small GTPases Rab5 and Rac1.

EXPERIMENTAL PROCEDURES

Strains and Reagents—Bacterial strains used in this study were DH5α (Invitrogen), HMS174 (DE3) (Novagen, Milwaukee, WI), and BL21RIL (DE3) (Stratagene, La Jolla, CA). Strains were grown in Luria-Bertani medium supplemented with ampicillin and kanamycin as needed (30). Vent DNA polymerase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA), Roche Molecular Biochemicals, and Invitrogen. [^{3}H]GDP was from PerkinElmer Life Sciences. Ni-NTA-agarose and penta-His antibody were from Qiagen Inc. (Valencia, CA). Amylose resin was purchased from New England Biolabs (Beverly, MA). Glutathione-Sepharose, CNBr-activated Sepharose, and protein A-Sepharose were from Amersham Biosciences. Monoclonal antibody to GST was a generous gift of Drs. Michael Brown and Joseph Goldstein (University of Texas Southwestern Medical Center, Dallas, TX). Monoclonal Rab5, microtubule-associated protein 2b (MAP2b), and EEA1 antibodies were from BD Biosciences (San Diego, CA). Monoclonal synaptophysin antibody was from Sigma. Monoclonal Rac antibody was purchased from Upstate Cell Signaling Solutions (Waltham, MA). Rhodamine-conjugated phalloidin and Alexa488/594-conjugated rabbit and mouse secondary antibodies were from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences. SuperSignal West Femto Sensitivity substrate was from Pierce Biotechnology Inc. All other products were purchased from Sigma unless otherwise noted.

Plasmid and Viral Constructions—His$_6$-Rab5 (a, b, and c), His$_6$-Rab4, and His$_6$-Rab11 *Escherichia coli* expression constructs and Rab5A:S34N, 5B:S34N, and 5C:S34N-PVJL11 two-hybrid bait constructs were described previously (29). RFP-Rab5a mammalian expression constructs were a generous gift of Dr. Richard Pagano (Mayo Foundation, Rochester, MN). The coding sequence for Ypt1 was inserted into pQE30 (Qiagen Inc., Valencia, CA) for creation of His$_6$-Ypt1p. Wild-type Rac1, Rac3, cdc42, and RhoA GST-tagged *E. coli* expression constructs were kind gifts of Drs. Paul Sternweis and Mike Rosen (University of Texas Southwestern Medical Center, Dallas, TX). The Vps9p domain of alsin (alsin-(1360–1657)) was PCR-amplified from a KIAA1563 partial clone (Kazusa DNA Research Institute, Kisarazu, Chiba, Japan) and subcloned into pMBP-parallel 1 (31), pET28b (Novagen, Inc., Milwaukee, WI), pGADGH (32), and pEGFP-C3 (Clontech, Palo Alto, CA) for creation of the MBP, His$_6$, two-hybrid prey, and GFP fusion constructs. A fragment of alsin containing the DH and PH domains (alsin-(685–1026)) was PCR-amplified from the KIAA1563 partial clone and subcloned into pFASTBacHTb (Invitrogen) and pEGFP-C3. To generate full-length alsin, the 5′ region of alsin was obtained by RT-PCR on RNA derived from the SH-SY5Y human neuroblastoma cell line (a kind gift of Dr. Martin Reick, University of Texas Southwestern Medical Center, Dallas, TX). The remainder of the gene was PCR-amplified from the KIAA1563 partial clone. These two fragments were digested and subcloned into pFASTBacHTa (Invitrogen) by three-piece ligation to generate full-length alsin. This plasmid was used as template in subsequent PCR reactions to amplify the NH$_2$-RCC1-like domain (RLD; alsin-(1–705)), a fragment of alsin consisting of the RLD, DH, and PH domains (alsin-(1–1026)), a fragment of alsin lacking the last 55 amino acids (ΔVps9p, alsin-(1–1602)), and full-length alsin for subcloning into pFASTBacHTb (Invitrogen), pACCMVpLpA (33), and/or pEGFP-C3 (Clontech, Palo Alto, CA). Recombinant alsin bacmids were generated in DH10BAC *E. coli* using the Bac-to-Bac baculovirus expression system (Invitrogen) and transfected into *Spodoptera frugiperda (*Sf9) cells. Recombinant alsin adenovirus was constructed using methods described previously (34). Propagation and titration of the adenovirus were as described previously (35). Viral stocks were kept between 10^8 and 10^9 plaque-forming units/ml and stored at −80 °C for later use.

Protein Purification—His$_6$-Rab5 (a, b, and c), His$_6$-Rab4, and His$_6$-Ypt1 constructs were transformed into HMS174 (DE3) *E. coli*. Cells were grown at 37 °C to an A_{600} of 0.6 and induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 5 h at 37 °C. Cells were harvested and proteins purified using Ni-NTA-agarose according to the instructions from the manufacturer. GST-Rho GTPase fusions and MBP-Vps9p domain (alsin-(1360–1657)) were transformed into HMS174 (DE3) *E. coli*. Cells were grown at 37 °C to an A_{600} of 0.5–0.6, shifted to 25 °C, and induced with 0.3 mM IPTG for 10–15 h. Cells were harvested and proteins purified using glutathione-Sepharose or amylose resin according to the protocols from the manufacturer. Recombinant His$_6$-alsin-(685–1026) (fragment consisting of DH and PH domains) was purified from 200 ml of baculovirus-infected Sf9 cell lysates using Ni-NTA-agarose as described. All proteins were buffer-switched or dialyzed into appropriate buffers, concentrated, and used immediately or stored at −80 °C for later use. Protein concentrations were determined by Bradford assay (Bio-Rad), and purity was estimated by Coomassie Blue staining of SDS-PAGE gels.

Antibody Production—His$_6$-Vps9p domain (alsin-(1360–1657)) was transformed into BL21 RIL (DE3) *E. coli*. Cells were grown at 37 °C to an A_{600} of 0.5–0.6, switched to 30 °C, and induced with 0.5 mM IPTG for 5–6 h. With these conditions, His$_6$-alsin-(1360–1657) was found exclusively in inclusion bodies. To isolate protein, cells were lysed, and the lysate was centrifuged at 13,000 × *g*. The resulting pellet containing the inclusion bodies was resuspended in SDS-sample buffer (20% glycerol, 10% β-mercaptoethanol, 6% SDS, 125 mM Tris (pH 6.8), 0.02% bromphenol blue), separated by SDS-PAGE, and the band corresponding to the His$_6$.alsin-(1360–1657) was excised. Protein was concentrated and used to immunize a New Zealand White rabbit as described previously (36). CNBr-activated Sepharose was coupled to purified MBP-alsin-(1360–1657) (described above) and used to affinity-purify alsin antibodies from antiserum according to the instructions from the manufacturer (Amersham Biosciences).

Cell Culture, Transfections, and Infections—Hippocampal neuron cultures from E18 Sprague-Dawley rats were prepared and maintained as described previously (37). NIH3T3 cells and SH-SY5Y cells were maintained in a 37 °C, 5% CO_2 environment and cultured in Dulbecco's modified Eagle's medium (Invitrogen) and Dulbecco's modified Eagle's/F-12 (1:1) media (HyClone Laboratories, Logan, UT), respectively, supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml). NIH3T3 cells were plated at various densities on 12-mm glass coverslips a day prior to all experiments. Cells were transfected with 0.2–0.5 μg of GFP-alsin constructs and 0.3–5 μg of RFP-Rab5A constructs using FuGENE-6 (Roche Molecular Biochemicals) or LipofectAMINE-2000 (Invitrogen) reagents according to the instructions from the manufacturers, and processed for immunofluorescence ~24 h later. For SH-SY5Y infections, cells were plated on 6-cm plates (Corning, Corning, NY) and infected the next day with 10–25 plaque-forming units/cell recombinant alsin or empty adenovirus in minimal media (lacking serum). After 2 h, the medium was aspirated and cells were fed with media containing serum. Infections were continued for an additional 18–24 h prior to experimentation.

Cerebellum Fractionation—One fresh rat cerebellum from a female E18 Sprague-Dawley rat was isolated and resuspended by Dounce homogenization in 5 ml of lysis buffer (0.32 M sucrose, 5 mM Tris (7.5), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 1× protease inhibitor mixture (*N*-tosyl-L-phenlalanine chloromethyl ketone, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, phenylmethylsulfonyl fluoride, leupeptin, trypsin inhibitor)). DNA was sheared by passage through an 18-gauge needle repeatedly, and the lysate was pelleted at 500 × *g* for 10 min to remove unlysed cells and debris. The supernatant was separated into four 0.5-ml aliquots and used in fractionations as described previously (38, 39). All four aliquots were centrifuged at 10,500 × *g* for 15 min. For one of the aliquots, the supernatant (S2) and pellet (P2) were isolated. The supernatant from a second aliquot was then pelleted at 165,000 × *g* for 2 h, generating S3 (supernatant) and P3 (pellet) fractions. For the other two aliquots, the pellets from the 10,500 × *g* spin were resuspended in 50 μl of lysis buffer, and hypotonically lysed by the addition of 450 μl of H_2O (with 1× protease inhibitor mixture) and passage through an 18–20-gauge needle 10 times. This mixture was then centrifuged at 25,000 × *g* for 20 min, generating LS1 (supernatant) and LP1 (pellet) fractions. The supernatant from this spin was then pelleted at 165,000 × *g* for 2 h, generating LS2 (supernatant) and LP2 (pellet) fractions. Supernatants were added to 125 μl of 5× SDS-sample buffer (0.312 M Tris (pH 6.8), 10% SDS, 25% β-mercaptoethanol, 0.05% bromphenol blue), and pellets were resuspended in 625 μl of SDS-sample buffer. Samples were then boiled at 95 °C for 5 min and analyzed by SDS-PAGE and Western blotting.

Binding and Guanine Nucleotide Exchange Assays—Yeast two-hybrid assays were performed as described (27, 29). Rab guanine nucleotide exchange assays were performed essentially as described previously (27, 29). Recombinant proteins used in assays were all purified as described above. For time-course exchange assays, 200 pmol of Rab5 (a, b, and c) was incubated with either 600 pmol of bovine serum albumin (BSA) or 600 pmol of MBP-Vps9p domain of alsin, and exchange was monitored at 0, 2, 5, 10, 20, and 30 min. In experiments aimed at determining Rab specificity, 100 pmol of Rab was incubated with 300 pmol of BSA or 300 pmol of MBP-Vps9p domain and GDP release measured at 0 and 30 min.

In vitro binding assays were performed essentially as described (40). Briefly, 5 μg of GST-Rho or His_6-Rab GTPases were added to 50 μl of glutathione-Sepharose or Ni-NTA-agarose resins. The reaction was brought up to 400 μl in H_2O and incubated end-over-end at 25 °C for 1 h. The beads were pelleted and resuspended in 500 μl of buffer (20 mM Tris (pH 7.5), 1 mM dithiothreitol, 10 mM EDTA, 50 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1× protease inhibitor mixture) and incubated end-over-end for 1 h at 25 °C to deplete the GTPases of nucleotide. During this incubation, 1 μg of His_6-alsin-(685–1026) (Rho binding assays) or alsin containing SH-SY5Y lysate (Rab binding assays) was incubated with 500 μl of buffer at 4 °C for 1 h. The beads were pelleted, resuspended in 500 μl of buffer, and added to the 500-μl buffer-alsin mixture. The reaction was incubated at 25 °C for 1 h end-over-end. The beads were then pelleted, washed twice with 1 ml of buffer, resuspended in 50 μl of SDS-sample buffer, and analyzed by SDS-PAGE and Western blotting with GST and His antibodies.

In Vivo Rac-GTP Loading Assay—A fusion construct (GST-PBD) consisting of GST and the Rac/cdc42 GTPase binding domain of p21-activated kinase family (PAK) (a generous gift of Dr. Paul Sternweis, University of Texas Southwestern Medical Center, Dallas, TX) was transformed into HMS174 (DE3) *E. coli*. Cells (200 ml) were grown at 37 °C to an A_{600} of 0.6 and induced with 0.3 mM IPTG for 3 h at 37 °C. Cells were harvested and resuspended in lysis buffer (20 mM HEPES (pH 7.5), 120 mM NaCl, 10% glycerol, 2 mM EDTA, 1× protease inhibitor mixture), sonicated twice for 15 s, and centrifuged for 30 min at 27,000 × *g*. The supernatant was adjusted to 0.5% Nonidet P-40 and incubated with 300 μl of glutathione-Sepharose beads end-over-end at 4 °C for 1 h. The GST-PBD glutathione-Sepharose conjugates were pelleted and washed five times in lysis buffer + 0.5% Nonidet P-40 and three times in lysis buffer without Nonidet P-40.

Sf9 cells (10–20 ml) were co-infected with full-length alsin baculovirus (described above) and Rac baculovirus (a generous gift of Dr. Paul Sternweis). After 48 h, cells were harvested and resuspended in 2 ml of buffer A (50 mM Tris (pH 7.5), 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM $MgCl_2$, 1× protease inhibitor mixture). Cells were lysed by Dounce homogenization and passage through a 22-gauge needle, and the resulting lysates were cleared at 16,000 × *g* for 10 min. Bradford assay was used to determine the protein concentration of the lysates, and 2 mg of total protein was incubated with 50 μg of glutathione-Sepharose-conjugated GST-PBD in 1 ml total volume (buffer A) end-over-end at 4 °C for 1 h. Beads were pelleted, washed four times with buffer B (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$, 1× protease inhibitor mixture), and resuspended in 75 μl of SDS-sample buffer. Proteins were eluted by boiling at 95 °C for 5 min, and sample was analyzed by SDS-PAGE and Western analysis with antibodies to Rac.

Immunoblotting—Samples were separated by SDS-PAGE (8–12%) and transferred to nitrocellulose. Blots were incubated with antibodies to alsin (1:500–1:1000), GST (1:2000), Rac (1:2000), synaptophysin (1:2000), and His (1:2000) followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000). Blots were developed by enhanced chemiluminescence using SuperSignal West Femto Sensitivity substrate (Pierce Biotechnology Inc.) and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY).

Immunofluorescence—Hippocampal neurons were processed for immunofluorescence as described previously (37). Primary antibodies were incubated at dilutions of 1:50 (alsin) and 1:250 (MAP2b) and secondary antibodies (Alexa488 anti-rabbit and Alexa568 anti-mouse) at 1:1000. Images were captured by laser scanning confocal microscopy (Zeiss LSM 510) using a 63× objective with an optical section of 1.5 μm. Images were prepared using Photoshop 7.0 (Adobe; San Jose, CA). For direct immunofluorescence of GFP and RFP fusion proteins, NIH3T3 cells were fixed in 4% formaldehyde (Tousimis, Rockville, MD) for 20 min and coverslips mounted using the Prolong antifade reagent (Molecular Probes, Eugene, OR). For indirect immunofluorescence, cells were fixed with 4% formaldehyde, permeabilized for 3 min with 0.1% Triton X-100, and blocked for at least 60 min in phosphate-buffered saline + 3% BSA. Primary antibodies were added for at least 60 min at dilutions of 1:50 (alsin) and 1:100 for Rac, Rab5, and EEA1. Secondary antibodies (Alexa488 anti-rabbit and Alexa568 anti-mouse) and rhodamine-conjugated phalloidin were added at 1:1000 for 60 min. Coverslips were mounted on slides using the Prolong antifade reagent (Molecular Probes). NIH3T3 images were captured by a Zeiss Axiovert S1002TV fluorescence microscope (fluorescein isothiocyanate and rhodamine filters) and Photometrix digital camera. Images were deconvoluted with Delta Vision software (Applied Precision, Inc., Issaquah, WI) and prepared using Photoshop 7.0 (Adobe, San Jose, CA).

RESULTS

RCC1 Repeats of Alsin—Alsin is predicted to be a relatively large protein of 1657 amino acids (13, 14) (see Fig. 1*A*). Comparison of alsin with other known proteins uncovered the presence of several sequence motifs including RCC1, DH, PH, MORN, and Vps9p domains (13, 14) (see Fig. 1*A*). The RCC1 motif derives its name from the RCC1 protein, a guanine nucleotide exchange factor for the Ran GTPase (20). RCC1 domains comprise ~50 amino acids that adopt a β-sheet conformation (21). In the case of the RCC1 protein, seven of these domains are tandemly arranged to comprise a seven-bladed propeller (41). It has been reported that alsin contains three (13) or six (14) RCC1 repeats, and it has been suggested that alsin may also possess Ran guanine nucleotide exchange activity (13). Close inspection of the NH_2-terminal portion of alsin identified five subdomains that fit the consensus RCC1 motif (21). Although over 90 known proteins contain one or more RCC1 repeats (21), only RCC1 itself has been shown to be a Ran GEF (20). Because of the diversity of proteins that contain RCC1 repeats, it is likely that this motif does not serve an enzymatic role, but instead functions as a protein-protein interaction motif. As expected, secondary structure prediction using the JPred algorithm (42) showed that the region of alsin containing the RCC1 repeats consists primarily of β-sheet with a central α-helix (Fig. 1*B*). Despite the fact that it contains only five RCC1 domains, there is ample β-sheet present to form a seven-bladed propeller, making alsin a likely member of the β-propeller family of proteins (43).

Alsin Stimulates Guanine Nucleotide Exchange on Rab5—Alsin is predicted to have a number of domains implicated in protein trafficking and cell signaling events (13, 14, 19) (see Fig. 1*A*). Three examples of these domains are the central DH and PH domains and the COOH-terminal Vps9p domain. Because the disease-associated mutations in *ALS2* are recessive and predicted to generate truncated proteins lacking some or all of the aforementioned domains (13, 14, 16–18), it is likely that these domains play an essential role in alsin function. We examined the biochemical activities of these domains, starting with the Vps9p domain.

The four proteins examined to date that contain Vps9p domains all exhibit guanine nucleotide exchange activity toward the Vps21p/Rab5 family of small GTPases (27–29, 44). They function to activate these GTPases, which in turn regulate vesicle trafficking events through the endocytic pathway. To determine whether the Vps9p domain of alsin possesses Rab5 nucleotide exchange activity, we first determined whether it interacted with Rab5. Both yeast two-hybrid and *in vitro* binding assays were used in these analyses. For the yeast two-hybrid experiments, the Vps9p domain of alsin (alsin-(1360–1657); see Fig. 1*A*) was fused to the Gal4 activation domain (prey) and co-expressed with various Rab5 LexA DNA binding domain fusions (baits). Interaction between bait and prey drives *HIS3* expression allowing the yeast transformants to grow in the absence of histidine. Both wild-type and a mutant form of Rab5 that is in the GDP-bound or nucleotide-free form (S34N) (29) were tested for their ability to interact with the Vps9p domain of alsin (Fig. 2*A*). All three isoforms of Rab5 (a, b, and c) were tested in this manner. Yeast expressing both the alsin-(1360–1657) prey and the S34N Rab5 baits were prototrophic for histidine, indicating that alsin interacted with the mutant Rab5 in its GDP-bound or nucleotide-free form (Fig. 2*A*). Yeast expressing wild-type Rab5 (*WT*) or empty LexA DNA binding domain fusions (baits) were unable to grow in the absence of histidine (*panel 1*), indicating that the Vps9p domain of alsin did not interact or interacted poorly with these baits. All of the strains were able to grow on media supple-

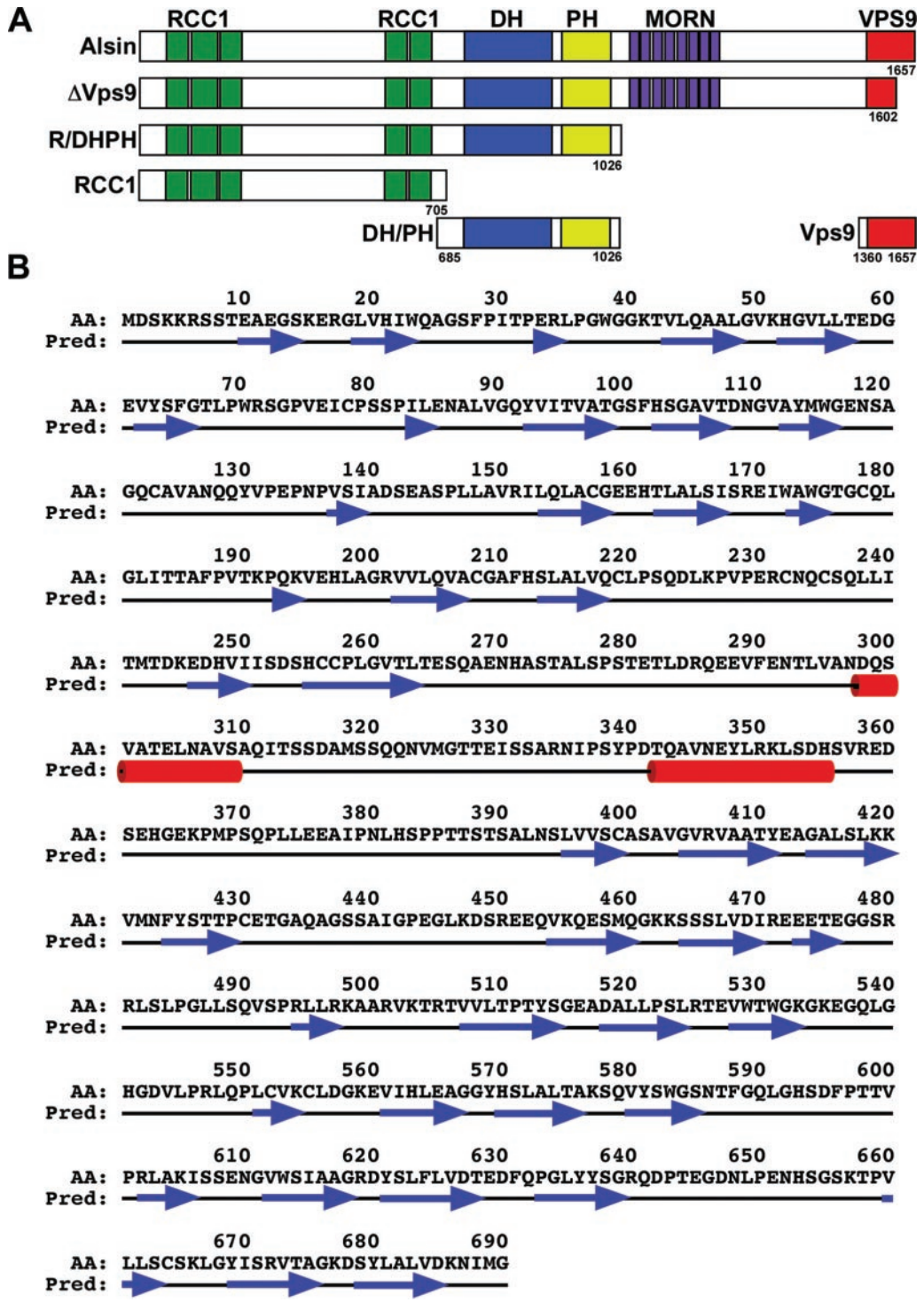

FIG. 1. **Secondary structure of alsin and truncations used in this study.** *A*, alsin possesses NH_2-terminal RCC1 repeats (*green*), central DH (*blue*) and PH domains (*yellow*), MORN repeats (*purple*), and a COOH-terminal Vps9p domain (*red*). Various fragments of the protein used in this study are indicated with the corresponding amino acids noted. *B*, the amino-terminal region of alsin containing the RCC1 repeats was analyzed using the secondary structure prediction algorithm, JPred (42). The *blue arrows* indicate sequences predicted to adopt a β sheet conformation, and the *red barrels* indicate predicted α helixes.

mented with histidine (Fig. 2*A*, *panel 2*). Full-length alsin prey was also observed to interact with the S34N Rab5 bait isoforms (data not shown).

To confirm the yeast two-hybrid result, *in vitro* binding experiments were performed with recombinant proteins. SH-SY5Y cell lysates overexpressing full-length alsin were incubated in the presence of His-tagged nucleotide-free Rab5a or Rab11, and the resulting complexes were precipitated with Ni-NTA-agarose. As shown in Fig. 2*B*, alsin bound His_6-Rab5a (*lane 4*) but not His_6-Rab11 (*lane 3*). Together, the *in vivo* two-hybrid and the *in vitro* binding assays indicated that alsin and Rab5 specifically interact.

To determine whether alsin binding to Rab5 stimulated guanine nucleotide exchange activity, *in vitro* nucleotide exchange assays were performed. Purified recombinant Rab5a was preloaded with ^{3}[H]GDP, and nucleotide release was monitored in the presence or absence of alsin. For these experiments the Vps9p domain of alsin (alsin-(1360–1657)) was expressed and

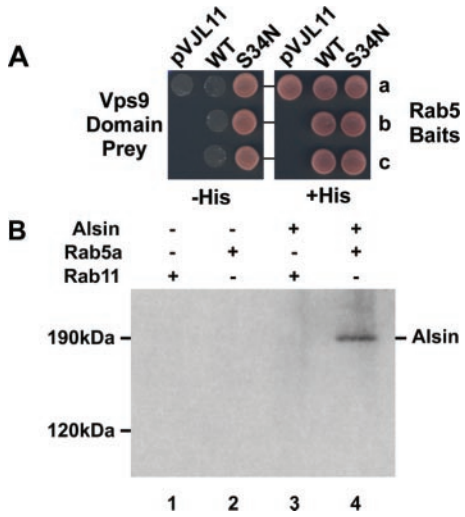

FIG. 2. **Alsin interacts with Rab5.** *A*, the Vps9p domain of alsin interacts with Rab5 by yeast two-hybrid. The Vps9p domain of alsin (alsin-(1360–1657)) fused to the Gal4 activation domain was co-expressed in L40 yeast with Rab5 isoforms (a, b, and c) fused to the LexA DNA binding domain (*WT* and S34N) or the LexA DNA binding domain alone (pVJL11). Interaction was scored as strain growth in the absence of histidine (*−His*). *B*, full-length alsin interacts with Rab5. Lysates from SH-SY5Y cells infected with an adenovirus coding for alsin or a control adenovirus were incubated in the presence of 5 μg of His_6-Rab11 or 5 μg of His_6-Rab5a as described under "Experimental Procedures." The Rab GTPases were isolated by the addition of Ni-NTA-agarose, and the presence of alsin was determined by SDS-PAGE and Western analysis with antibodies to alsin.

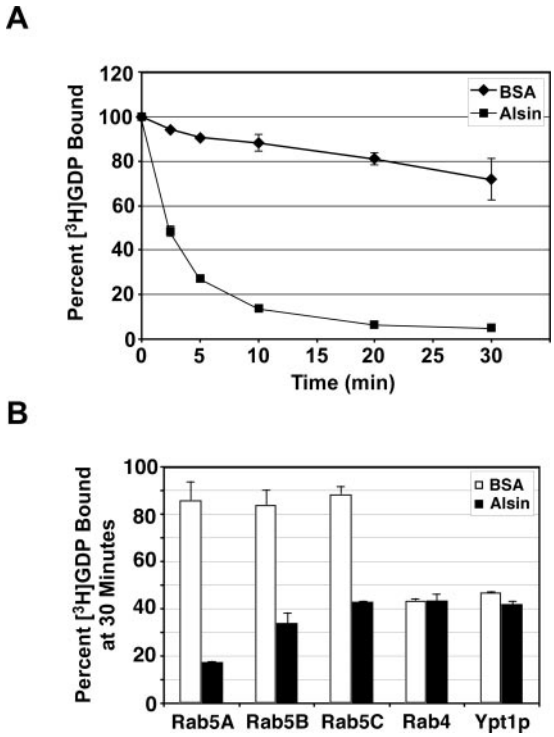

FIG. 3. **Alsin is a Rab5 guanine nucleotide exchange factor.** *A*, alsin stimulates GDP release on Rab5a. The Vps9p domain of alsin (alsin-(1360–1657)) was expressed and purified as an MBP fusion protein from *E. coli*. 200 pmol of His_6-Rab5a was preloaded with 0.7 μM [^{3}H]GDP for 30 min at 30 °C. Samples were then incubated in the presence of 600 pmol of BSA or 600 pmol of MBP-alsin-(1360–1657), and excess unlabeled nucleotide and [^{3}H]GDP release was monitored over time by nitrocellulose filtration and scintillation counting. Samples were normalized to the ^{3}H count at 0 min. Shown is the average of two independent experiments with *error bars* corresponding to one standard deviation. *B*, alsin guanine nucleotide exchange activity is specific for Rab5. 100 pmol of His_6-Rab GTPases were preloaded with [^{3}H]GDP as described above, and release was monitored in the presence of 300 pmol of BSA or 300 pmol of MBP-alsin-(1360–1657). Results shown correspond to the amount of [^{3}H]GDP-bound His_6-Rab after 30-min incubation with alsin (or BSA) normalized to the amount at 0 min and are the average of two independent experiments with *error bars* representing one standard deviation.

purified from *E. coli* as an MBP fusion protein. As seen in Fig. 3*A*, alsin-(1360–1657) greatly stimulated GDP release on Rab5a. More than 75% of Rab5a released its associated GDP in the presence of the alsin Vps9p domain by 5 min. In comparison, when BSA was added to the reaction instead of alsin-(1360–1657), only 10% of the Rab5a had displaced GDP by 5 min, indicative of the low intrinsic activity exchange associated with Rab5a (28, 29). Exchange factors for the Rab GTPase family exhibit strict substrate specificity. To demonstrate that this exchange activity was indeed specific to Rab5, nucleotide exchange assays were performed with other Rab GTPases (Fig. 3*B*). The Vps9p domain of alsin was unable to stimulate GDP release on the Rabs, Rab4, and Ypt1p. Because the Vps9p domain of alsin interacted with all three isoforms of Rab5 by yeast two-hybrid (Fig. 2*A*), we also tested whether it catalyzed nucleotide release on Rab5b and c isoforms. Alsin was active on all three Rab5 isoforms *in vitro* (Fig. 3*B*), although highest activity was observed on Rab5a.

Alsin Is a Rac1 Exchange Factor—In addition to its Vps9p domain, alsin possesses central DH and PH domains. This tandem repeat has been shown previously to catalyze guanine nucleotide exchange on members of the Rho family of GTPases (22). Unlike Rab exchange factors, Rho GEFs are promiscuous and substrate specificity cannot be easily determined by computational analysis. Therefore, we first set out to identify Rho family members that interacted with alsin. A His_6-tagged alsin fragment containing the DH and PH domains (alsin-(685–1026), see Fig. 1*A*) was expressed and partially purified from Sf9 cells. This fragment was then used in *in vitro* binding assays with various Rho GTPases expressed in *E. coli* as GST fusions. The GST-Rho GTPases were complexed with glutathione-Sepharose, incubated with the DH/PH domains of alsin, and the potential complexes were isolated. As shown in Fig. 4*A*, alsin-(685–1026) interacted specifically with Rac1 (*lane 2*). No or very little interaction was observed with the related Rho family members Rac3, RhoA, or cdc42 (*lanes 3–5*) or with GST alone (*lane 1*). To address whether Rac1 interacts with alsin in the context of the full-length protein, similar binding reactions were performed with Sf9 cell lysates overexpressing full-length alsin. Full-length alsin copurified with GST-Rac1 but not with GST or glutathione-Sepharose alone (data not shown).

The ability of alsin to stimulate Rac1 nucleotide exchange was tested using an *in vivo* assay. This method utilizes the fact that proteins of the PAKs interact with Rac1 only when it is in the active GTP-bound state. Using a GST-PAK fusion protein complexed with glutathione-Sepharose beads, GTP-bound Rac1 can be specifically isolated from cell lysates and quantified (45, 46). To examine Rac1 activation by alsin, Rac1 and alsin were co-expressed in Sf9 cells, cell lysates were generated, and GTP·Rac1 was complexed to GST-PAK. The GTP·Rac1·GST-PAK complexes were then isolated, resolved by SDS-PAGE, and the amount of Rac1·GTP bound was determined by densitometry. Shown in Fig. 4*B* is a representative example of this analysis. Coexpression of alsin with Rac1 resulted in a significant increase in the levels of activated Rac1 (*lower panel*, *lane 3*) when compared with Rac1 alone (*lower panel*, *lane 2*). The average of six independent experiments showed that alsin coexpression resulted in a ~4-fold increase in relative Rac1·GTP levels (normalized to total Rac1). This value was determined to be statistically significant using a one-sam-

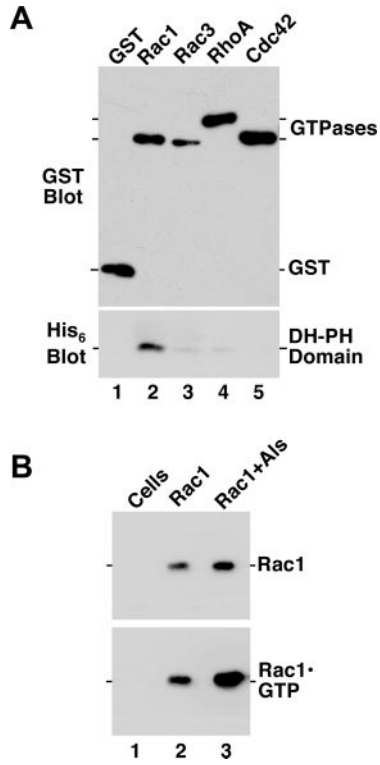

FIG. 4. **Alsin is a Rac1 guanine nucleotide exchange factor.** *A*, alsin interacts specifically with Rac1. A fragment of alsin consisting of the DH and PH domains (alsin-(685–1026)) was expressed and partially purified as a His_6 fusion from Sf9 cells. 5 μg of the indicated GST-Rho GTPases were conjugated to glutathione-Sepharose beads and incubated in the presence of alsin-(685–1026). Beads were isolated and the resulting complexes analyzed by SDS-PAGE and Western blotting with antibodies to His_6 (to identify alsin) and GST (to identify the Rho family member). *B*, alsin stimulates GTP loading on Rac1 *in vivo*. Sf9 cells expressing both Rac1 and full-length alsin or Rac1 alone were lysed and supernatants incubated in the presence of GST-PBD (Rac GTPase binding domain of PAK) as described under "Experimental Procedures." GST-PBD binds specifically to activated Rac1 allowing isolation of the GTP-bound species. GST-PBD complexes were precipitated and analyzed by SDS-PAGE and Western blotting with antibodies directed against Rac1 (*lower panel*). Small fractions of whole cell lysates corresponding to 1–3% of the input used for the pull-down were analyzed to determine the relative levels of total Rac1 in each lysate (*upper panel*). The amount of Rac1·GTP precipitated was quantified by densitometry and normalized to the amount of Rac1 in each lysate. Shown is one example of the six experiments used to determine a mean 3.9 ($\pm$ 2.2)-fold increase ($p = 0.02$, using a one-sample t test) in Rac1·GTP when alsin was coexpressed with Rac1.

ple t test ($p = 0.02$; see Fig. 4 legend). These results demonstrated that alsin stimulated Rac1 guanine nucleotide exchange activity *in vivo*.

Alsin Localizes to Punctate Membrane Structures—To gain insight into the potential role of alsin in the maintenance of neuron function, we examined its subcellular localization. Rat embryonic hippocampal neurons were isolated and cultured *in vitro*, and endogenous alsin localization was determined by indirect immunofluorescence. The anti-alsin antibodies used were directed against the COOH-terminal Vps9p domain of the protein. This polyclonal antiserum recognized a protein of the expected size for alsin in an extract of rat cerebellum (Fig. 5*A*), which has been shown to be an enriched source of alsin (13). Preimmune serum did not cross-react with this protein species. The alsin antiserum also specifically reacted with a unique 190-kDa polypeptide in Sf9 and SH-SY5Y cells that were expressing full-length recombinant alsin (data not shown). These data demonstrate that this antiserum specifically recognizes alsin.

When affinity-purified alsin antibodies were used to localize alsin in rat embryonic hippocampal neurons, the protein was found primarily on small punctate structures (Fig. 5*B* and *insets*). Additionally, some cytoplasmic staining was also observed. Specific decoration of dendrites with MAP2b antibody (*upper panels*) revealed that alsin was present in dendrites, axon, and the cell body, with no apparent polarized localization. Localization was independent of time in culture, as a similar pattern was seen at both 7 and 14 days. To ensure that the pattern observed was indeed rat alsin, we preincubated the alsin antiserum with antigenic peptide (MBP-alsin-(1360–1657)). Addition of the peptide effectively blocked the signal observed, indicating that the signal is specific to alsin (Fig. 5*B*, *right panels*).

To further characterize the localization of alsin, fractionation experiments were performed on extracts from rat cerebellum. As shown in Fig. 5*C*, alsin is present in both soluble and pelletable pools, with an enrichment in the P3 fraction (see *lane 3*). This fraction corresponds to the microsomal or small membranes, and Rab5 has been shown to be enriched in P3 as well (Ref. 47 and data not shown). Alsin is also found in the LP1 fraction, indicating that a portion of the protein is associated with synaptosomal membranes. To ensure that the membrane integrity was not perturbed by the fractionation procedure, we followed the pattern of the synaptic protein synaptophysin. Synaptophysin was found completely in membrane fractions, similar to previous observations (48), indicating that membrane structures were not disturbed by this protocol.

Alsin Colocalizes with Rab5 and Stimulates Endosome-Endosome Fusion—Because alsin demonstrated guanine nucleotide exchange activity toward Rab5 (Fig. 3), the localization pattern of alsin was compared with that of Rab5. As shown in Fig. 6*A*, alsin and Rab5 partially colocalized to small punctate membrane structures throughout NIH3T3 cells. These structures are likely endosomal, as alsin was also observed to colocalize with EEA1 (early endosome antigen 1), a known marker of early endosomes (Fig. 6*B*).

To examine whether alsin was capable of activating Rab5 *in vivo*, we took advantage of the fact that Rab5 activation stimulates endosome-endosome fusion, resulting in endosome enlargement (49, 50). Wild-type Rab5a and the Vps9p domain of alsin were co-expressed in NIH3T3 cells as red fluorophore (RFP) and green fluorophore (GFP) proteins, respectively, and endosome dynamics were monitored by immunofluorescence. Overexpression of the Vps9p domain of alsin and wild-type Rab5a dramatically altered the appearance of Rab5a-positive endosomal structures (Fig. 6*C*). Instead of the small Rab5-positive punctate structures seen with overexpression of Rab5 alone, greatly enlarged endosomal structures were seen. This pattern has also been observed when an activated form (GTPase-deficient) of Rab5 (29, 50, 51) or the Rab5 exchange factor Rabex-5 (data not shown) is expressed in cells. The formation of the Rab5a-positive enlarged endosomes was dependent upon wild-type Rab5a and alsin expression, as they were largely absent upon co-expression of dominant-negative Rab5a (Fig. 6*D*) or with wild-type Rab5a alone (data not shown). Interestingly, other structures were also seen, which contained only the Vps9p domain of alsin (Fig. 6*C*). The exact nature of this compartment is unknown.

Alsin Colocalizes with Rac in Membrane Ruffles and Lamellipodia—In the course of experimentation, it was observed that the endogenous alsin staining pattern exhibited a dependence on cell density. When cells were plated at lower densities that promote cell migration, alsin was present both at leading membrane edges and in a punctate staining pattern throughout the cell cytoplasm (Fig. 7). Partial colocalization between alsin,

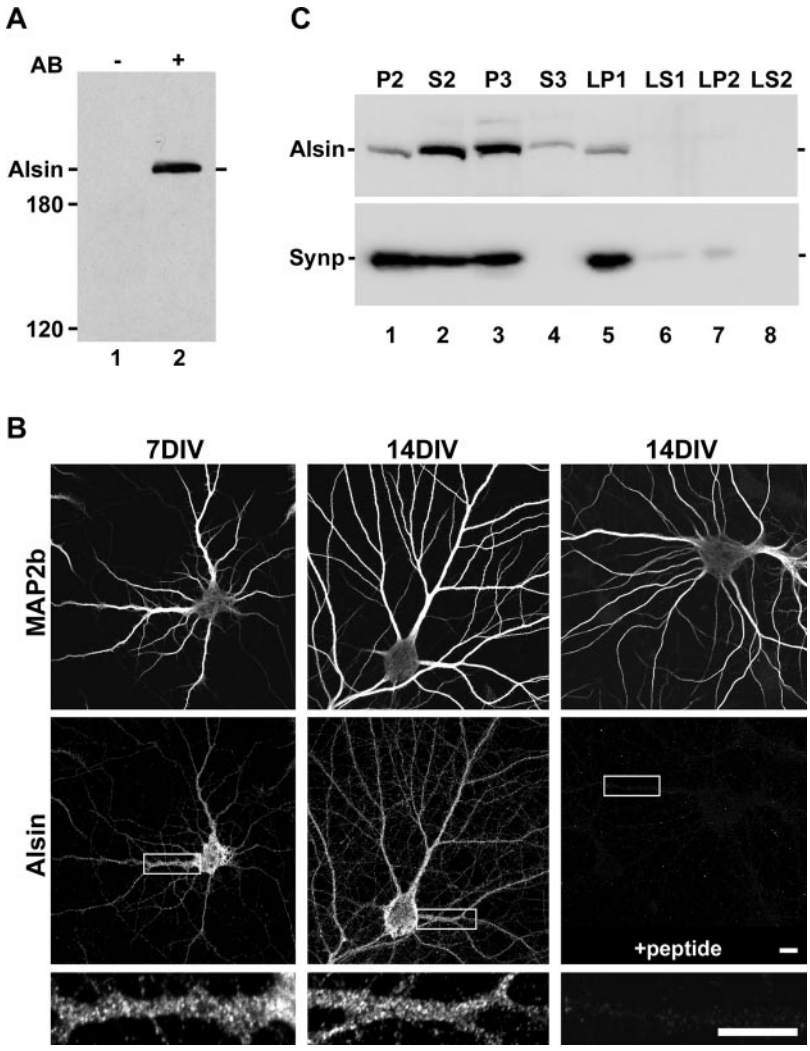

FIG. 5. **Alsin localizes to punctate membrane structures.** *A*, alsin antiserum immunoprecipitates alsin from rat cerebellum. Rat cerebellum lysate was incubated in the presence of affinity-purified alsin (*lane 2*) or preimmune antisera (*lane 1*) and precipitated by the addition of protein A-Sepharose. Immunoprecipitated material was separated by SDS-PAGE, and the presence of alsin was determined by Western blot analysis. *B*, alsin localizes to cytoplasmic punctate membrane structures in hippocampal neurons. Rat embryonic hippocampal neurons were isolated and maintained *in vitro* for 7 days (*panel A*) or 14 days (*panels B* and *C*). Endogenous alsin was detected by indirect immunofluorescence with polyclonal antiserum directed against alsin. Dendrites were visualized by staining with MAP2b antibody. The *bottom panel* shows a higher magnification of the box indicated. Preincubation of the alsin antibodies with antigenic peptide (alsin-(1360–1657), *panel C*) effectively competes away the signal indicating the specificity of the antibody. Cells shown are representative images of the overall population. *C*, alsin is present in cytoplasm and membrane fractions in rat cerebellum. An extract of rat cerebellum was generated and fractionated as described under "Experimental Procedures." The presence of alsin and synaptophysin in the various fractions was determined by immunoblot analysis.

Rab5 (Fig. 6*A*), and Rac (Fig. 7*A*) to these punctate structures was also observed. More striking, however, was the overlap of alsin and Rac at membrane ruffles. In many cells, colocalization at these sites was complete. Because Rac is known to play a role in actin remodeling at these sites (23–25), we asked whether alsin and actin colocalized in membrane ruffles and lamellipodia. NIH3T3 cells expressing low levels of GFP-tagged alsin were stained with rhodamine-conjugated phalloidin to label the actin cytoskeleton. As shown in Fig. 7 (*B* and *C*), alsin was present in actin-positive membrane ruffles and lamellipodia. Alsin colocalized with actin in membrane ruffles in two other cell types as well (data not shown). Although alsin localized to sites of actin remodeling, overexpression of alsin alone did not seem to stimulate these events (data not shown).

Alsin Domain Localization—To determine which domain(s) of alsin were involved in membrane localization, a number of alsin-GFP fusion constructs consisting of one or more domains were expressed in NIH3T3 cells and their subcellular localization was determined by fluorescent microscopy. Overexpression of the full-length alsin-GFP construct showed a difference in localization when compared with endogenous alsin. Although a portion of overexpressed alsin was found in punctate membrane structures similar to those containing endogenous alsin, the majority of overexpressed protein was observed primarily in the cytosol (Fig. 8*A*). The alsin subdomain-GFP fusions were then used to dissect potential subcellular localization signals. The NH_2-terminal RLD (alsin-(1–705), Fig. 1*A*) of alsin was found almost exclusively in the soluble cytoplasmic fraction, whereas a major portion of the DH/PH (alsin-(685–1026), Fig. 1*A*) and Vps9p (alsin-(1360–1657), Fig. 1*A*) domain GFP fusion proteins were found primarily associated with intracellular structures (Fig. 8, *C* and *D*). The Vps9p-GFP domain localized to membrane structures (Fig. 8, *B* and *D*) that are likely enlarged endosomes similar to those observed upon overexpression of Rab5 (Fig. 6*C*) or another Rab5 exchange factor, Rabex-5 (data not shown). The DH/PH-GFP (alsin-(685–1026), Fig. 1*A*) domains fractionation pattern revealed that this domain was present in bright punctate structures (Fig. 8, *B* and *C*). The exact nature of these structures is unknown, but they are reminiscent of an endosomal staining pattern. Mutations in *ALS2* that lead to the disease phenotype have been found throughout the gene and are predicted to encode prematurely truncated protein products (13, 14, 16–18). It can be inferred from these mutations that alsin protein lacking the Vps9p domain is nonfunctional. To begin to determine the effects that loss in the Vps9p domain has on alsin function, we made two additional GFP fusion constructs of alsin lacking complete Vps9p domains and analyzed their subcellular localization. One of the fragments lacked the MORN and Vps9p domains and comprises the RCC1 repeats and DH/PH domains (alsin-(1–1026); see Fig. 1*A*) only. The other fragment was prematurely truncated at amino acid 1602 deleting approximately half of the Vps9p domain (ΔVps9p; alsin-(1–1602); see Fig. 1*A*). The ΔVps9d construct closely resembles a recently identified mutational allele in ALS2 (18). Both alsin-GFP fusions that lacked a functional Vps9 domain resembled were

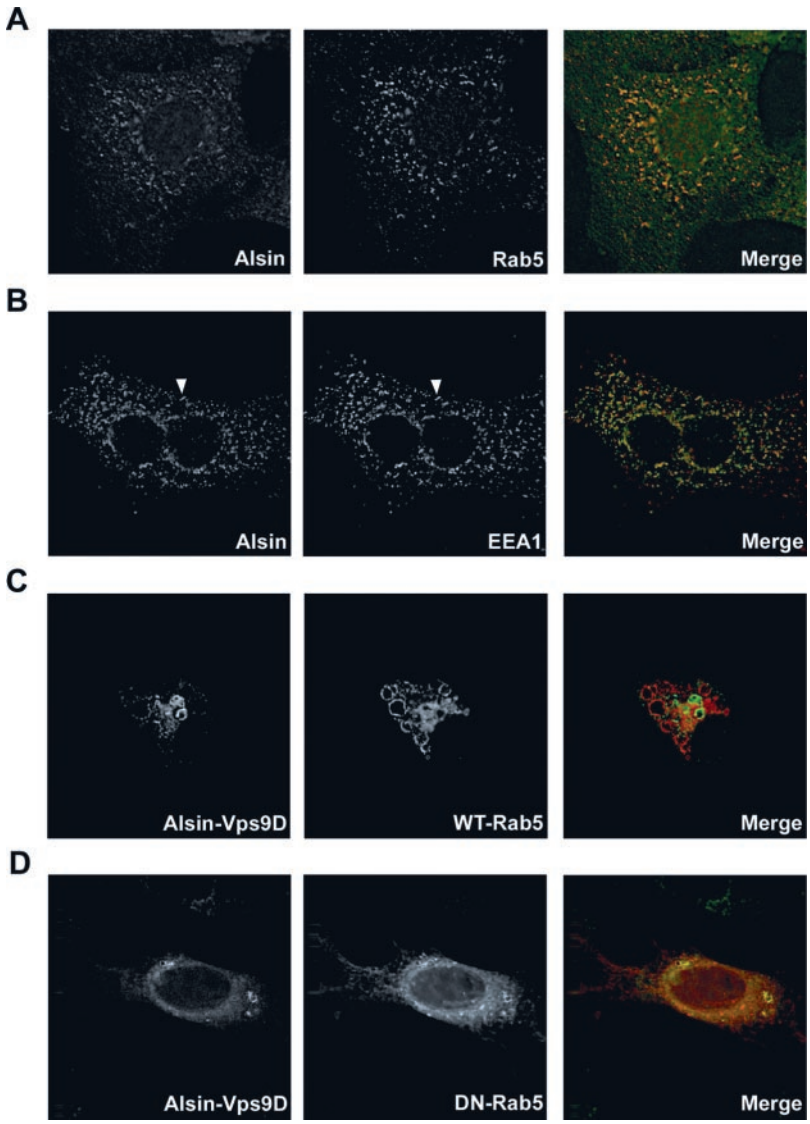

FIG. 6. **Alsin colocalizes with Rab5 and stimulates endosome-endosome fusion.** *A*, endogenous alsin colocalizes with Rab5. Approximately 80–90% confluent NIH3T3 cells were decorated with antibodies to alsin and Rab5. *B*, endogenous alsin colocalizes with EEA1. Approximately 80–90% confluent NIH3T3 cells were decorated with antibodies to alsin and EEA1. *C*, the Vps9p domain of alsin stimulates endosome-endosome fusion. Subconfluent cells were co-transfected with GFP-Vps9p domain of alsin and wild-type RFP-Rab5a and processed for microscopy 20–24 h later. *D*, dominant-negative Rab5a blocks endosome-endosome fusion. Subconfluent cells were co-transfected with GFP-Vps9p domain of alsin and dominant-negative RFP-Rab5a as in *C*.

largely cytoplasmic (Fig. 8, *E* and *F*), similar to that seen with full-length alsin (Fig. 8*A*). These data suggest that alsin has multiple localization signals that are able to contribute to cytoplasmic and/or membrane distribution.

DISCUSSION

The *ALS2* gene product is a large protein that possesses many functional domains that are implicated in cell signaling and membrane transport events. We show here that the Vps9p domain of alsin mediated an interaction with Rab5 and acted as a specific guanine nucleotide exchange factor for Rab5 *in vitro* and *in vivo*. Similar to other Vps9p domain-containing proteins, alsin exhibited exquisite specificity for Rab5 and did not enhance nucleotide release on the other Rab proteins tested. Alsin also showed a slight preference for the Rab5a isoform, but the significance of this observation is unclear. Recently, the Rab5 GEF activity of alsin has been reported to require the presence of the MORN repeats in addition to the Vps9p domain (52). The results presented here demonstrate that the Vps9p domain alone can catalyze Rab nucleotide exchange. The specificity of alsin action was also reflected by its colocalization with Rab5 on punctate structures in fibroblasts. A similar staining pattern was also observed with EEA1, suggesting that alsin-positive structures are early endosomal in nature. When the localization of endogenous alsin was examined in hippocampal neurons, the same punctate staining pattern was also observed. Overexpression of the Vps9p domain of alsin with Rab5a resulted in the enlargement of Rab5a-positive structures, similar to the phenotype observed upon overexpression of an activated mutant of Rab5. Interestingly, the alsin-Vps9p domain fragment also was present on enlarged endosomal-like compartments that were not decorated by Rab5. These alsin-Vps9p domain-positive membranes may represent a unique endosomal structure. Further characterization of these compartments will be needed to define the potential role they may have in alsin function.

In addition to its COOH-terminal Vps9p domain, alsin possesses centrally located DH and PH domains, which are a hallmark of guanine nucleotide exchange factors of the Rho GTPase family (22). It was found that a fragment of alsin containing these domains (alsin-(685–1026)) interacted specifically with Rac1 and that alsin activated Rac1 *in vivo*, generating the GTP-bound form of the GTPase. Unfortunately, we were unable to observe *in vitro* guanine nucleotide exchange activity on Rac1 with alsin-(685–1026) alone (data not shown). This may be because of the requirement of a cofactor or post-translational modification on alsin to promote exchange activity. This phenomenon has been observed with a number of Rac exchange factors, including Ect2, mammalian Son-of-Sevenless (mSos), P-Rex1, SWAP-70, and Dock180 (53–58). With each of these GEFs, phosphorylation or interaction with other proteins

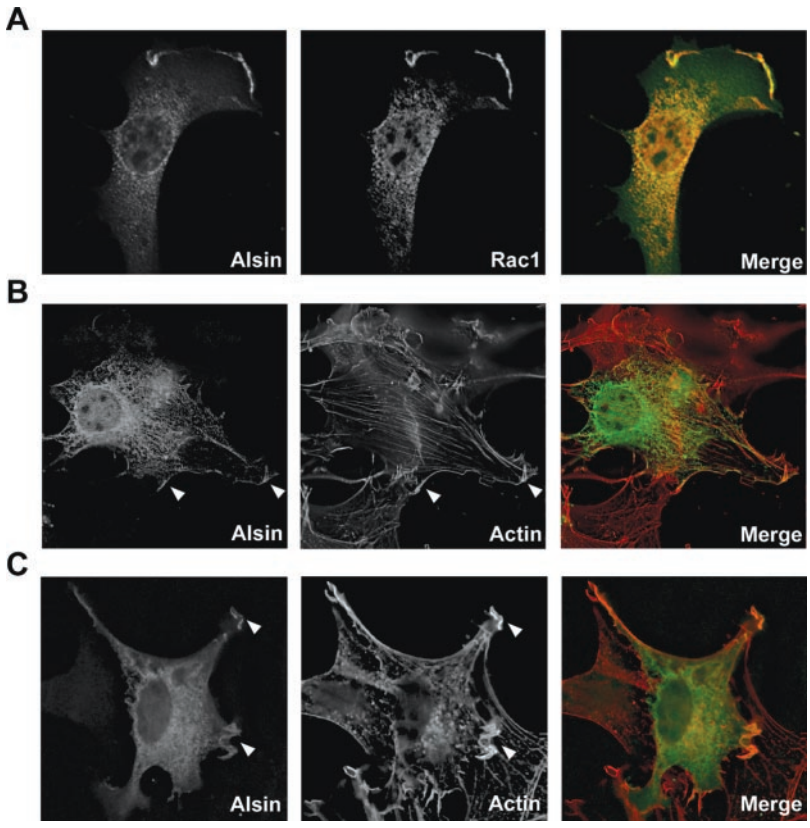

FIG. 7. **Alsin colocalizes with Rac in membrane ruffles and lamellipodia.** *A*, endogenous alsin colocalizes with Rac. Approximately 30–40% confluent NIH3T3 cells were stained with antibodies to alsin and Rac1. At lower densities that promote cell migration, alsin is observed at leading membrane edges in addition to the perinuclear region seen in cells at higher densities. Importantly, Rac and alsin colocalize at both of these sites, with a marked enrichment at membrane edges. *B* and *C*, alsin colocalizes with membrane ruffles and lamellipodia. GFP-tagged alsin was transfected into ~30–40% confluent NIH3T3 cells and processed for immunofluorescence 20–24 h later. Cells were decorated with rhodamine-phalloidin to label the actin cytoskeleton.

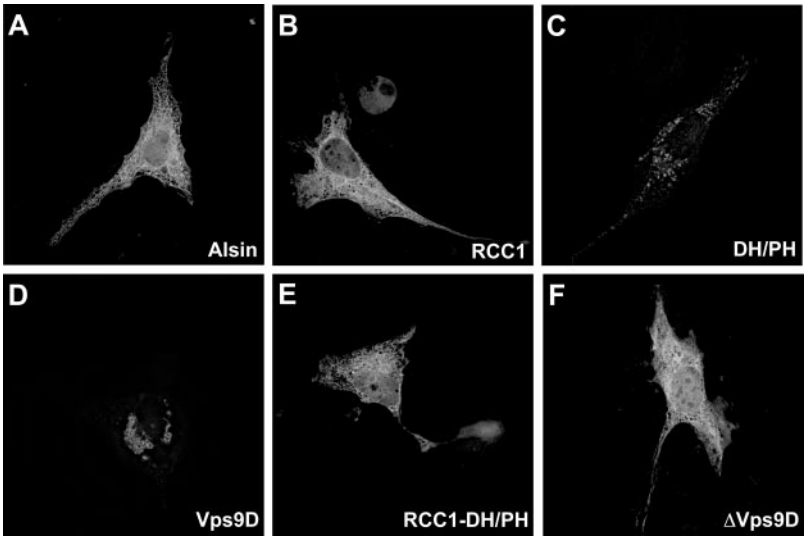

FIG. 8. **Localization of overexpressed alsin subdomains.** Subconfluent NIH3T3 cells were transfected with full-length alsin fused to GFP (*A*), or GFP fusion constructs containing the alsin RLD (*B*), the DH/PH domains (*C*), the Vps9p domain (*D*), the RLD/DH/PH domains (*E*), or alsin lacking the COOH-terminal half of the Vps9p domain (*F*). The fragments used are also shown in *A*. The cells were processed for immunofluorescence 24 h later. Cells shown are representative of the overall population.

and/or phosphatidylinositol 3,4,5-trisphosphate was shown to be required for maximal nucleotide exchange activity *in vitro*. Another intriguing aspect of these results is the specificity of the interaction of alsin with Rac1. Though closely related, Rac3 showed little or no interaction with alsin. This was somewhat surprising considering the high level of sequence identity shared by these two family members. However, differences in the distribution of Rac1 and Rac3 in the brain have been reported (59), as well as their ability to interact with downstream effectors to promote cell spreading and cell adhesion (60). The alsin-Rac1 interaction may reflect an additional level of functional specificity among the Rac isoforms.

In addition to the DH, PH, and Vps9p domains, alsin possesses other domains that are likely to play an important functional role. For instance, the NH_2-terminal region of alsin consists of five RCC1 repeats. Computational analysis predicts that this region is capable of adopting a seven-bladed propeller structure similar to that observed in RCC1 (Ref. 20; see Fig. 1*B*). It was originally suggested that this NH_2-terminal region of alsin may function as a Ran GEF, because of its similarity to RCC1 and the presence of the RCC1 repeats (13). Because of the ubiquitous presence of RCC1 repeats in many proteins of diverse function, it is likely that this region of alsin is of structural importance rather than enzymatic importance. The potential propeller of alsin may serve as a protein-protein interaction surface similar to the function of the seven-bladed propeller heterotrimeric G protein β subunit (61, 62). Perhaps interaction with a cytoplasmic protein through this domain sequesters alsin in the cytoplasm. This may play a role in regulating the Rac1 and Rab5 guanine nucleotide exchange activity of alsin. Additionally, the β propeller domain may bind back and interact with another domain of alsin, serving to

allosterically inhibit the Rac1 and Rab5 GEF domains. This may be reflected by the fact that overexpression of full-length alsin was unable to stimulate Rab5-mediated endosome-endosome fusion (data not shown), whereas the Vps9p domain alone possesses this activity. Further characterization of the β propeller domain and the proteins that interact with it should provide insight into how it may regulate alsin function.

How could a protein that specifically activates Rac1 and Rab5 be important for motor neuron maintenance? Several hypotheses can be proposed. First, alsin may be involved in motor neuron maintenance by regulating actin dynamics. Actin has been shown to be involved in a number of processes in neurons, many of which provide structural integrity and include: dendritic spine formation (63–65) and plasticity (reviewed in Ref. 66), activity-dependent formation of new active presynaptic terminals (67), axon guidance (reviewed in Ref. 68), and cadherin-catenin regulation of synaptic structural plasticity (69, 70). Actin has also been shown to be involved in receptor trafficking events in neurons, such as clustering of post-synaptic receptors (71, 72), recycling of endocytic vesicles and subsequent transport to the synaptic vesicle cluster (73), transport of exocytic synaptic vesicles (74), and regulation of synaptic vesicle fusion (75). Many processes that involve actin function are known to be regulated by Rac (23–25). Activation of Rac by alsin could stimulate actin remodeling observed in all of the aforementioned processes. Furthermore, it has been suggested that Rab5 may play a role in the formation of membrane ruffles (76). Therefore, alsin guanine nucleotide exchange activity on Rac1 or Rab5 could provide key upstream regulation of the actin pathway. An intriguing hypothesis is that alsin activation of Rac1 and Rab5 are temporally distinct events resulting in transient but separate stimulation of actin dynamics. It is now being appreciated that perhaps as many as four different actin remodeling events are required for endocytosis (77). Alsin, through Rac1 and Rab5 activation, may serve to regulate many of these events.

A second role for alsin in motor neuron maintenance could involve the regulation of glutamate receptor endocytosis. It has been hypothesized that elevated levels of glutamate at the synaptic cleft trigger excessive activation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors, the subsequent activation of *N*-methyl-D-aspartate receptors and Ca^{2+} influx (78). Motor neuron death as a result of ALS is generally thought to be apoptotic in nature, as mouse models show upstream caspase activation followed by cytochrome *c* release from the mitochondria (79–83). Moreover, inhibition of caspases and cytochrome *c* release and overexpression of anti-apoptotic proteins of the Bcl-2 family can prolong survival in these models (79, 80, 84). An overwhelming amount of evidence exists showing that α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, *N*-methyl-D-aspartate, and metabotropic (mGlu) glutamate receptors are cleared from the cell surface by endocytosis (85–102). Rab5 activation is required for the endocytosis of many plasma membrane receptors. Alsin regulation of Rab5 would provide a key point of regulation in the endocytic pathway. In addition, as mentioned above, alsin activation of Rac1 and Rab5 could serve to regulate the actin dynamics required for endocytosis and other cellular functions. For example, stimulus-induced dendritic arbor growth not only requires glutamate receptor mediated synaptic transmission, but also an increase in Rac activity and modulation of the actin cytoskeleton (103, 104). If alsin was involved in the endocytosis of glutamate receptors, a loss in alsin function could be expected to cause an abundance of glutamate receptors at the cell surface, and subsequently, excessive activation through these receptors, triggering apoptosis.

Third, a loss of alsin function may negatively impact neurotrophic factor signaling. Signaling events that initiate at distal dendrites and axons must be propagated to the cell body to mediate their effects on cellular homeostasis (105, 106). These signaling intermediates, termed "signaling endosomes," have been shown to contain Rab5 (107). In addition, Rac activation has been observed in neurotrophic receptor signaling cascades (108). Therefore, through its regulation of Rac1 and Rab5, alsin may be involved in the formation and maturation of neurotrophic receptor-containing signaling endosomes. In the absence of functional alsin, internalization of activated receptors and their participation in neuronal retrograde signaling to the cell body may be lost, eventually leading to cellular apoptosis.

Because the disease-causing mutations in *ALS2* suggest that the Vps9p domain alone is required for alsin function, the above hypotheses focus on pathways that require Rab5 and involve Rac1. However, a more central role for Rac1 activation in alsin function should also be considered. In addition to its established role in regulating the actin cytoskeleton, Rac1·GTP also interacts with p35/cdk5 kinase (109). Although the functional significance of this interaction is not clearly understood, p35/cdk5 is required for neurite outgrowth (110) and synaptic vesicle endocytosis (111), implicating a role for Rac1·GTP in both of these processes. Additionally, although it is well appreciated that phosphatidylinositides activate Rac1 through direct interaction with the PH domain of Rac1 GEFs, recent evidence has been presented that shows Rac1·GTP can positively regulate phosphatidylinositide production through its interaction with phosphatidylinositol 3-kinase (112). This suggests a potential role for alsin in the production of signaling lipids. Finally, Rac2·GTP has been implicated in the generation of reactive oxygen species by regulating the assembly of the NADPH oxidase (113). Although primarily utilized in phagocytic leukocytes (Rac2), Rac1-mediated reactive oxygen species production has now been observed in other cellular contexts and is thought to play a role in cell signaling (113). Therefore, alsin Rac1 GEF activity may potentially link these processes and/or actin cytoskeleton remodeling to membrane trafficking events regulated by the Vps9p domain. Further studies will be required to determine which of these cellular events is perturbed when alsin function is lost in *ALS2*, juvenile-onset primary lateral sclerosis, and infantile-onset hereditary spastic paralegia.

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