Promotion of Neurite Extension by Protrudin Requires Its Interaction with Vesicle-associated Membrane Protein-associated Protein

Shotaro Saita1,2, Michiko Shirane2, Tohru Natume3, Shun-ichiro Iemura2, and Keiichi i. Nakayama3

From the 1Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan, 2CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan, and the 3National Institutes of Advanced Industrial Science, Kohtoh-ku, Tokyo 135-0064, Japan

Protrudin is a protein that contains a Rab11-binding domain and a FYVE (lipid-binding) domain and that functions to promote neurite formation through interaction with the GDP-bound form of Rab11. Protrudin also contains a short sequence motif designated FFAT (two phenylalanines in an acidic tract), which in other proteins has been shown to mediate binding to a FYVE (lipid-binding) domain and that functions to promote neurite formation through interaction with the GDP-bound form of Rab11. Protrudin also contains a short sequence motif designated FFAT (two phenylalanines in an acidic tract), which in other proteins has been shown to mediate binding to vesicle-associated membrane protein-associated protein (VAP). We now show that protrudin associates and colocalizes with VAP-A, an isoform of VAP expressed in the endoplasmic reticulum. Both the interaction between protrudin and VAP-A as well as the induction of process formation by protrudin were markedly inhibited by mutation of the FFAT motif. Furthermore, depletion of VAP-A by RNA interference resulted in mislocalization of protrudin as well as in inhibition of neurite outgrowth induced by nerve growth factor in rat pheochromocytoma PC12 cells. These defects resulting from depletion of endogenous rat VAP-A in PC12 cells were corrected by forced expression of (RNA interference-resistant) human VAP-A but not by VAP-A mutants that have lost the ability to interact with protrudin. These results suggest that VAP-A is an important regulator both of the subcellular localization of protrudin and of its ability to stimulate neurite outgrowth.

The molecular mechanisms that underlie neurite formation include both cytoskeletal remodeling and membrane trafficking. Membrane components are transported in a directional manner within the cell by a membrane recycling system, resulting in expansion of the surface area of the neurite. The small GTPase Rab11 regulates membrane recycling and constitutive exocytosis (1), and it is thought to contribute to neurite formation through regulation of directional membrane transport.

We have recently identified protrudin as a key regulator of Rab11-dependent membrane trafficking during neurite exten-
sion. Protrudin interacts with FKBPs (also known as FKBPs) (2), which is a member of the immunophilin family of proteins that bind the immunosuppressant drug FK506 (3). FKBPs are multifunctional proteins that regulate the folding or export of other proteins as a result of their peptidyl-prolyl cis-trans-isomerase activity (4). Protrudin was found to interact with...
lacks the membrane-spanning domain. VAP-A and VAP-B share ~60% amino acid sequence identity, form homo- or heterodimers, and are expressed in many tissues (14–16). In addition to their localization to the ER (16), VAP-A and VAP-B are present in a wide range of intracellular membranes or membrane structures, including the Golgi, the ER-Golgi intermediate compartment (17), tight junctions (18), neuromuscular junctions (19), recycling endosomes, and the plasma membrane (20).

We have now identified VAP-A and VAP-B as proteins that interact with protrudin. Protrudin preferentially interacts with VAP-A via its FFAT motif, and this motif was found to be required for the protrudin-dependent formation of membrane protrusions in HeLa cells. In addition, depletion of VAP-A by RNA interference resulted in inhibition of NGF-induced neurite outgrowth in the PC12 rat pheochromocytoma cell line. This inhibition of neurite outgrowth was reversed by expression of human VAP-A but not by that of VAP-A mutants that have lost the ability to bind to protrudin. These results suggest that interaction of protrudin with VAP-A is important both for its ER retention and for its ability to stimulate neurite formation.

### EXPERIMENTAL PROCEDURES

#### Construction of Plasmids

Construction of vectors encoding human protrudin and FKBP52 was described previously (2, 21). Complementary DNAs encoding mutants of protrudin were generated by the PCR with Prime Star polymerase (Takara, Ohtsu, Japan); those encoding VAP-A or VAP-B were generated by PCR from a human kidney cDNA library; and that encoding RAMP4 was generated by PCR from a mouse thyroid cDNA library. The VAP or RAMP4 cDNAs were subcloned into the pEF-BOS-2×HA or pEF-BOS-2×MYC vectors (kindly provided by Shigekazu Nagata, Kyoto University, Japan) or into pGEX-6P (Amersham Biosciences). DNA fragments encoding stem-loop-type short hairpin RNAs (shRNAs) specific for rat T-complex protein 1, δ subunit (TCPD); T-complex protein 1, γ subunit (TCPG); T-complex protein 1, ε subunit (TCPE); VAP-A (VAP-A); VAP-B (VAP-B); and ATGGA-3'); or human VAP-B mRNA (5’-GGGAGGAGAAC-AAGCAGTCCA-3') were synthesized, attached to the U6 promoter, and subcloned into the vector pIRES-Venus-B (Venus cDNA was kindly provided by Atsushi Miyawaki, RIKEN, Japan), which contains an internal ribosome entry site and encodes the reporter protein Venus.

#### Protein Identification by Liquid Chromatography-MS/MS Analysis

Human protrudin tagged with the FLAG epitope at its NH₂ terminus was transiently expressed in HEK293T cells

### Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence coverage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicle associated membrane protein-associated protein A (VAP-A)</td>
<td>14.5</td>
</tr>
<tr>
<td>Vesicle associated membrane protein-associated protein B and C (VAP-B/C)</td>
<td>14.4</td>
</tr>
<tr>
<td>T-complex protein 1, δ subunit (TCPD)</td>
<td>9.2</td>
</tr>
<tr>
<td>T-complex protein 1, γ subunit (TCPG)</td>
<td>7.7</td>
</tr>
<tr>
<td>T-complex protein 1, ε subunit (TCPE)</td>
<td>6.3</td>
</tr>
</tbody>
</table>

### Figure 1

**Interaction between protrudin and VAP.** A, extracts of HEK293T cells transiently transfected with expression vectors for 3×FLAG-tagged protrudin or FKBP52 (negative control) and for 2×HA-tagged VAP-A or VAP-B were subjected to immunoprecipitation (IP) with anti-FLAG. The resulting precipitates, as well as a portion (1% of the input for immunoprecipitation) of the cell extracts, were subjected to immunoblot analysis (IB) with anti-HA, anti-FLAG, or anti-calnexin. B, mouse brain extract was subjected to immunoprecipitation with anti-protrudin or control rabbit IgG, and the resulting precipitates, as well as a portion (4% of the input for immunoprecipitation) of the tissue extract, were subjected to immunoblot analysis with anti-VAP-A and anti-protrudin. IgL, Ig light chain.

### Figure 2

**Role of the FFAT motif in the interaction of protrudin with VAP-A.** A, domain organization of human protrudin. B, extracts of HEK293T cells transiently transfected with an expression vector for 3×FLAG-tagged protrudin, protrudin mutants (ΔFFAT or D294A), or FKBP52 were subjected to immunoprecipitation with anti-FLAG. The resulting precipitates, as well as a portion (1% of the input for immunoprecipitation) of the cell extracts, were subjected to immunoblot analysis with anti-VAP-A, anti-FLAG, or anti-calnexin.
FIGURE 3. Delineation of the regions of VAP-A responsible for interaction with protrudin. A, domain organization of human VAP-A and structure of deletion mutants thereof. A summary of the ability of the mutants to bind protrudin as determined in B is shown on the right. B, full-length (FL) VAP-A or the mutants thereof shown in A fused at their NH2 termini to the 2× HA tag were expressed in HEK293T cells together with 3× FLAG-tagged protrudin or FKBP52. The cell extracts were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates, as well as a portion (1% of the input for immunoprecipitation) of the cell extracts, were subjected to immunoblot (IB) analysis with anti-HA, anti-FLAG, or anti-calnexin. C, full-length human VAP-A and the indicated mutants thereof fused at their NH2 termini to the 2× HA tag were expressed together with 3× FLAG-tagged protrudin in HEK293T cells. The cell extracts were subjected to immunoprecipitation with anti-FLAG, and the resulting precipitates, as well as a portion (1% of the input for immunoprecipitation) of the cell extracts, were subjected to immunoblot analysis with anti-HA, anti-FLAG, or anti-calnexin. D, structure of VAP-A mutants fused to GST at their NH2 termini and a summary of their ability to bind to a His6-tagged COOH-terminal fragment of protrudin comprising residues 207–409 (protrudin-C) as determined in E. E, His6-tagged protrudin-C was incubated with the GST-tagged VAP-A mutants shown in D or with GST-RhoA or GST as negative controls, and the binding mixtures were then subjected to precipitation with glutathione-conjugated beads. The bead-bound proteins (Pull-down), as well as a portion (3 or 10% of the input for precipitation) of the binding mixtures, were subjected to immunoblot analysis with anti-His6 and anti-GST. The asterisks indicate bands corresponding to the GST-tagged proteins.
Regulation of Protrudin Function by VAP

![Image](https://via.placeholder.com/350)

and then purified together with associated proteins from cell lysates by immunoaffinity chromatography with anti-FLAG. Protrudin-associated proteins were digested with Lys-C endoproteinase, and the resulting peptides were analyzed with a nanoscale liquid chromatography-MS/MS system as described previously (22, 23). The peptide mixture was applied to a MightySil-PR-18 (particle size, 1 μm; Kanto Chemical, Tokyo, Japan) fritless column (45 × 0.150-mm inner diameter) and fractionated over 30 min at a flow rate of 50 nl/min with a 0 to 40% gradient of acetonitrile in 0.1% formic acid. Eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-ToF Ultima; Micromass, Manchester, UK). Mass spectrometry and MS/MS spectra were obtained in a data-dependent mode. Up to four precursor ions with an intensity above a threshold of 10 counts/s were selected for MS/MS analysis from each survey scan. All of the MS/MS spectra were compared with protein sequences in Swiss Prot and RefSeq (NCBI) with the use of batch processes of the Mascot software package (Matrix Science, London, UK). The criteria for match acceptance included the following: (i) if the match score exceeded the threshold by 10, identification was accepted without further consideration; (ii) if the difference between the score and the threshold was <10, or if a protein was identified on the basis of a single matched MS/MS spectrum, we manually confirmed the raw data before acceptance; and (iii) peptides assigned by fewer than three y series ions and those with a charge of +4 were eliminated regardless of their scores.

Cell Culture and Transfection—HEK293T and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). PC12 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum; they were treated with recombinant human NGF (Millipore, Billerica, MA) at 100 ng/ml in RPMI 1640 supplemented with 1% fetal bovine serum. PC12 cells were transfected with the use of a Nucleofector system (Amaxa Biosystems, Cologne, Germany), whereas other cell types were transfected with the use of the FuGENE 6 reagent (Roche Applied Science).

Antibodies—Rabbit polyclonal antibodies (for immunoblot analysis and immunoprecipitation) and a mouse monoclonal antibody (for immunofluorescence staining) specific for protrudin were generated in response to a His<sub>6</sub>-tagged recombinant protein corresponding to amino acids 206–335 of human protrudin. A mouse monoclonal antibody to VAP-A was obtained from BD Biosciences; and a mouse monoclonal antibody to protein-disulfide isomerase (PDI) was from Affinity Bioreagents (Golden, CO). Alexa 488- or Alexa 546-conjugated goat antibodies to mouse or rabbit IgG were from Molecular Probes (Invitrogen).

Immunoblot Analysis and Immunoprecipitation—The cells were lysed in a solution containing 40 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 10 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 10 μM MG132. After incubation for 10 min at 4 °C, the lysate was centrifuged for 1 min at 4 °C. The crude microsomal pellet was resuspended in a solution containing 40 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 10 μM MG132, incubated for 10 min at 4 °C, and then centrifuged at 20,400 × g for 1 min at 4 °C. The protein concentration of the resulting supernatant was determined with the Bradford assay (Bio-Rad). Whole mouse brain was homogenized by 10 strokes (900 rpm) of a Potter homogenizer in a solution containing 20 mM HEPES-NaOH (pH 7.6), 0.32 M sucrose, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM NaF, aprotinin (10 μg/ml), leupeptin (10 μg/ml), 10 μM MG132, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The homogenate was centrifuged at 1000 × g for 5 min at 4 °C, and the resulting supernatant was centrifuged at 60,000 × g for 2 h at 4 °C. The crude microsomal pellet was resuspended in a solution containing 40 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 10 μM MG132, incubated for 10 min at 4 °C, and then centrifuged at 20,400 × g for 1 min at 4 °C. The protein concentration of the resulting supernatant was then determined with the Bradford assay.

The cell and mouse brain extracts were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were visualized by staining with Coomassie Blue. Immunoblot analysis was performed with the use of antibodies as described above. The signals were quantified by densitometry and normalized to actin or tubulin.
were transferred to an Immobilon-P membrane (Millipore) and probed with primary antibodies. Immune complexes were detected with Super Signal reagents (Thermo Scientific, Rockford, IL). For immunoprecipitation, cell or mouse brain extracts were incubated with anti-FLAG or anti-protrudin and with protein G-Sepharose 4 Fast Flow (Amersham Biosciences).

**Immunofluorescence Staining**—HeLa cells grown on glass coverslips were transfected with the use of the FuGENE 6 rea-

---

**FIGURE 5. Role of the MSP domain in the interaction of VAP-A with protrudin.** A, structure of human VAP-A, the MSP mutant thereof, RAMP4, and the chimeric protein MSP-RAMP4TM as well as a summary of their abilities to bind protrudin as determined in C. B, left panel, HeLa cells expressing 2×Myc-tagged VAP-A, MSP, RAMP4, or MSP-RAMP4TM were fixed and processed for immunofluorescence analysis with anti-Myc (green) and anti-PDI (red). The merged images are also shown. B, right panel, HeLa cells expressing 3×FLAG-protrudin and 2×Myc-tagged VAP-A, MSP, RAMP4, or MSP-RAMP4TM were fixed and processed for immunofluorescence staining with anti-FLAG (green) and anti-Myc (red). The merged images are also shown. C, VAP-A, MSP, RAMP4, or MSP-RAMP4TM fused at their NH₂ termini to the 2×Myc tag were expressed in HEK293T cells together with 3×FLAG-tagged protrudin or FKBP52. The cell extracts were subjected to immunoprecipitation (IP) with anti-FLAG, and the resulting precipitates, as well as a portion (1% of the input for immunoprecipitation) of the cell extracts, were subjected to immunoblot analysis with anti-Myc, anti-FLAG, or anti-calnexin. IB, immunoblot.
Regulation of Protrudin Function by VAP

gent and subsequently prepared for immunostaining. In brief, the cells were fixed for 15 min at room temperature with 4% formaldehyde in phosphate-buffered saline (PBS) and then incubated for 1 h at room temperature first with primary antibodies in PBS containing 0.1% bovine serum albumin and 0.1% saponin and then with Alexa 488- or Alexa 546-labeled goat secondary antibodies at a dilution of 1:2000. The cells were finally stained with Hoechst 33258 (Wako, Osaka, Japan), covered with a drop of GEL/MOUNT (Biomed, Foster City, CA) and examined with a confocal fluorescence microscope (Radiance 2000, Bio-Rad).

Transfected PC12 cells were grown on glass coverslips coated with poly-L-lysine and subsequently prepared for immunostaining. In brief, the cells were fixed for 10 min at room temperature with 4% formaldehyde in PBS and were then incubated for 1 h at room temperature first with primary antibodies in PBS containing 0.1% bovine serum albumin and 0.1% saponin and then with Alexa 546-labeled goat secondary antibodies at a dilution of 1:2000. The cells were then covered with a drop of GEL/MOUNT and examined with a confocal fluorescence microscope (Radiance 2000).

**In Vitro Binding Assay**—Recombinant GST- or His6-tagged proteins were expressed in and purified from *Escherichia coli*. Recombinant His6-protrudin(207–409) (1 μg in 12 μl of a solution containing 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and GST-VAP-A or mutants thereof (0.5 μg in 600 μl of a solution containing 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) were mixed and then incubated at 4 °C for 1 h with rotation. After the addition of glutathione-Sepharose 4B beads (Amersham Biosciences), the mixture was incubated for an additional 1 h at 4 °C with rotation. The beads were then washed twice with 50 volumes of Tris-buffered saline, and the bound proteins were subjected to immunoblot analysis.

**Quantitation of Process Formation**—Transfected cells with processes whose length was greater than the diameter of the nucleus were counted. For evaluation of the effects of FKBP52, protrudin, or protrudin mutants, the cells were immunostained with the M2 antibody to FLAG, whereas the effects of VAP-A or VAP-B were evaluated by immunostaining with the HA-Y11 antibody to HA. Ten nonoverlapping photomicrographs of each sample were examined for cell protrusion with a confocal fluorescence microscope (Radiance 2000).
Regulation of Protrudin Function by VAP

RESULTS

Protrudin Interacts with VAP-A via Its FFAT Motif—To investigate the physiological role of protrudin, we adopted a proteomics approach to identify proteins with which it is physically associated in cells. Human protrudin tagged with the HA epitope was expressed in HEK293T cells transfected with an expression vector for HA-tagged protrudin and subjected to immunoprecipitation assay with anti-HA and anti-FLAG. The immunoprecipitates were subjected to LC-MS/MS analysis and identified proteins such as VAP-A and VAP-B/C (Table 1). Together, these various data indicated that protrudin interacts with VAP-A in the physiological setting.

A similar analysis was performed to detect the potential interaction between endogenous proteins. Immunoprecipitates prepared from mouse brain extracts with anti-protrudin were subjected to immunoblot analysis with anti-VAP-A and anti-protrudin. Endogenous VAP-A was coprecipitated in the experimental conditions (Fig. 1B). In addition, the interaction of protrudin with VAP-A was confirmed in the cellular setting. Given that VAP interacts with the FFAT motif of certain proteins (11, 25), we examined whether the binding of VAP-A to protrudin might be dependent on the FFAT motif of protrudin. We constructed two mutants of human protrudin, ΔFFAT and D294A (Fig. 2A); the former lacks the entire FFAT motif (EFKDAIE), and the latter contains Ala instead of Asp294, which is required for binding of proteins to the FFAT motif (6). The amount of endogenous VAP-A associated with either mutant in HEK293T cells was greatly reduced compared with that associated with the wild-type protein, even though the expression levels of the protrudin mutants were similar to that of the wild-type protein (Fig. 2B). The residual interaction observed between VAP-A and the protrudin mutants was likely attributable to the fact that VAP and FFAT motif-containing proteins form a 2:2 tetramer (26), with the result that the protrudin mutants may be present in complexes of the endogenous wild-type protrudin with VAP-A. These data thus suggested that the interaction of protrudin with VAP-A is dependent on its FFAT motif.

VAP-A Interacts with Protrudin in the ER via Its MSP and TMs—VAP-A contains a major sperm protein (MSP) domain, a coiled-coil (CC) domain, and a single TM. We next investigated which of these domains are required for interaction with protrudin by generating a series of deletion mutants of VAP-A (∆MSP29, ∆MSP59, ∆MSP89, ∆MSP, ∆TM, ∆ER, and ∆CC) and examining their ability to associate with FLAG-protrudin in a coimmunoprecipitation assay (Fig. 3, A and B). Deletion of 59 amino acids from the NH2 terminus of VAP-A (∆MSP59) did not appear to affect its interaction with protrudin, whereas deletion of 89 amino acids from the NH2 terminus (∆MSP89) or of the entire MSP domain (∆MSP) greatly reduced the efficiency of such binding. The COOH-terminal transmembrane domain of VAP-A appeared essential for the interaction with protrudin, given that the deletion mutants MSP and ∆TM did not show binding. The COOH terminus of VAP-A contains a KDEL-like sequence (KFIL), which functions as an ER retention signal (27). A mutant (∆ER) that lacks this
sequence associated with protrudin, albeit with a reduced efficiency. The coiled-coil domain, which is thought to contribute to protein-protein interaction, was not necessary for binding of VAP-A to protrudin. We also prepared a series of point mutants of VAP-A. In particular, mutation of Lys<sup>94</sup> and Met<sup>96</sup> (equivalent to Lys<sup>87</sup> and Met<sup>89</sup> of the rat protein), residues that have been shown to be critical for binding to other FFAT motif-containing proteins, attenuated (but did not abolish) the interaction between VAP-A and protrudin (Fig. 3C), whereas other mutations (K52N, T53A/T54A, or K125N) corresponding to residues that are important for binding of the yeast VAP homolog Scs2p to other FFAT motif-containing proteins (28) did not affect the interaction of VAP-A with protrudin.

To examine the direct interaction between VAP-A and protrudin in vitro, we performed a pulldown assay. Recombinant GST-tagged VAP-A and six mutants thereof were produced in bacteria and tested for their ability to bind to recombinant His<sub>6</sub>-tagged protrudin (Fig. 3, D and E). The VAP-A mutants K94D/M96D, MSP, ΔMSP, and TM bound to protrudin, although the efficiency of binding appeared to be lower than that with full-length VAP-A. In contrast, the mutants ΔMSP/ΔTM and ΔMSP/ΔTM<sub>2</sub> (as well as the negative controls GST and GST-RhoA) did not interact with protrudin. These results suggested that both the MSP and transmembrane domains of VAP-A are required for binding to protrudin.

The VAP-A mutant MSP did not interact with protrudin in vivo, although it did so in vitro. One possible explanation of this discrepancy was that the subcellular distribution of the VAP-A mutant differs from that of protrudin. We therefore performed immunofluorescence analysis of HeLa cells expressing various HA-tagged VAP-A constructs and FLAG-tagged protrudin. The immunofluorescence signals of wild-type VAP-A as well as of VAP-A mutants that retain an intact transmembrane domain merged with those of the ER marker protein PDI (Fig. 4A) (29), suggesting that they reside in the ER. These VAP-A constructs also colocalized with protrudin (Fig. 4B). In contrast, VAP-A mutants that lack an intact transmembrane domain (MSP and ΔTM) were distributed throughout the cytoplasm and only partially colocalized with protrudin or PDI. The distribution of protrudin appeared to be in part independent of coexpressed VAP-A mutants, probably because a substantial amount of endogenous VAP-A is present in these cells; protrudin presumably associates with the endogenous VAP-A and thereby localizes to the ER. These results thus suggested that both the MSP domain and the transmembrane domain of VAP-A contribute to the interaction with the FFAT motif of protrudin and that the transmembrane domain is also necessary for ER retention, which is a prerequisite for the interaction with protrudin.

To examine whether the MSP domain of VAP-A is intrinsically capable of interacting with protrudin in vivo, we generated a chimeric protein, designated MSP-RAMP4TM (Fig. 5A). RAMP4 is a small tail-anchored protein that exposes the NH<sub>2</sub> and COOH termini to the cytoplasmic and lumenal sides of the ER membrane, respectively (30). MSP-RAMP4TM was constructed by fusion of the MSP domain of VAP-A and the COOH-terminal transmembrane domain of RAMP4. Immuno-
nolucene analysis of HeLa cells expressing FLAG-tagged protru-
din as well as Myc epitope-
tagged forms of VAP-A, MSP, 
RAMP4, or MSP-RAMP4TM re-
vealed that VAP-A, RAMP4, and 
MSP-RAMP4TM colocalized with 
the ER marker protein PDI (Fig. 5B), 
suggesting that these proteins reside 
in the ER. VAP-A and MSP-
RAMP4TM also colocalized with 
protrudin (Fig. 5B). We next exam-
ned the ability of MSP-RAMP4TM 
to associate with protrudin in 
a communoprecipitation assay. 
VAP-A and MSP-RAMP4TM inter-
acted with protrudin, whereas MSP 
and RAMP4 did not (Fig. 5C). These 
results thus suggested that the MSP 
domain of VAP-A alone is able to 
interact with protrudin if it is local-
ized to the ER. They are also consist-
ent with the in vitro binding data 
showing that the MSP domain of 
VAP-A associates with protrudin in 
solution (Fig. 3E).

**Mutation of the FFAT Motif 
Attenuates Protrudin Function—**
Expression of FLAG-protrudin in 
HeLa cells results in the generation 
of long processes (2) (Fig. 6A). To 
evaluate the effect of the interaction 
of protrudin with VAP-A on the 
process forming activity of protru-
din, we first examined process for-
mation in HeLa cells expressing 
FFAT mutants of protrudin 
(ΔFFAT and D294A) that have lost 
the ability to bind VAP-A. Protru-
usion was observed in <5% of cells 
expressing FKBP52 (negative con-
trol), whereas ~20% cells express-
ing wild-type protrudin showed 
process formation. In contrast, the 
efficiency of process formation was 
significantly reduced in cells 
expressing either FFAT mutant of 
protrudin compared with that in 
cells expressing the wild-type pro-
tein (Fig. 6, A–C). The residual 
process forming activity of the 
FFAT mutants was likely attribut-
able to the formation of VAP-pro-
trudin complexes containing both 
wild-type and mutant protrudin, 
as mentioned above. These results suggested that the FFAT 
motif of protrudin is necessary for the ability of the protein 
to induce process formation.

We next examined the effect of VAP overexpression on proc-
ess formation. Neither VAP-A nor VAP-B alone induced proc-
ess formation in HeLa cells (Fig. 6D). However, the expression
Regulation of Protrudin Function by VAP

Depletion of VAP-A results in an abnormal subcellular distribution of protrudin. A, PC12 cells were transfected for 48 h with an expression vector for VAP-A-shRNA 2 or VAP-B-shRNA 2 or with the corresponding empty vector (pIRES-Venus-B). The cell extracts were then prepared and subjected to immunoblot (IB) analysis with anti-VAP-A, anti-VAP-B, or anti-calnexin. B, PC12 cells transfected as in A were stimulated with NGF for 6 h and then subjected to immunofluorescence staining with anti-VAP-A, anti-VAP-B, or anti-PDI. Arrowheads indicate the pericentrosomal region. C, PC12 cells transfected as in A but in the additional presence of an expression vector for 3× FLAG-protrudin were stimulated with NGF for the indicated times and then subjected to immunofluorescence staining with anti-FLAG (red). The cells were also monitored for Venus fluorescence (green). D, HeLa cells were transfected for 48 h with an expression vector for VAP-A-shRNA 2 or for an shRNA specific for human VAP-B (or with the corresponding empty vector, pIRES-Venus-B) and then subjected to immunoblot analysis with anti-VAP-A, anti-VAP-B, or anti-calnexin. E, HeLa cells transfected as in D were stained with anti-FLAG (red) or anti-PDI (red). The cells were also monitored for Venus fluorescence (green). Arrows indicate cells depleted of VAP-A (Venus positive); the arrowhead indicates a cell not depleted of VAP-A (Venus negative). The merged images are also shown. F, a model of VAP-A function in the regulation of protrudin.

of VAP-A slightly but significantly increased the stimulatory effect of protrudin on process formation. VAP-B did not exhibit such an effect, consistent with the observation that the extent of binding of VAP-B to protrudin was much reduced compared with that of VAP-A (Fig. 1A).

We also examined the effect of VAP-A depletion on the process forming activity of protrudin by RNA interference with a VAP-A shRNA (VAP-A-shRNA 3) that targets both rat and human VAP-A mRNAs (Fig. 6E). Depletion of VAP-A resulted in marked inhibition of the process forming activity of protrudin (Fig. 6F). These results thus suggested that the interaction of protrudin with VAP-A is important for the process forming activity of protrudin.

VAP-A Is Essential for Protrudin-dependent Neurite Outgrowth—We next investigated whether VAP might be necessary for neurite extension in neurons by examining NGF-induced neurite outgrowth in PC12 cells depleted of VAP-A or VAP-B by RNA interference. Three different shRNAs specific for VAP-A mRNA or for VAP-B mRNA were tested for their ability to deplete the corresponding protein (Fig. 7A), and the most effective shRNAs (VAP-A-shRNA 2 and VAP-B-shRNA 2) were examined for their effects on NGF-induced neurite formation in PC12 cells. Depletion of VAP-A, but not that of VAP-B, resulted in marked inhibition of NGF-induced neurite outgrowth (Fig. 7, B and C). Moreover, depletion of VAP-A in PC12 cells eventually led to cell death (data not shown), as previously observed in rat primary neurons (31). These results thus suggested that VAP-A is essential for neurite formation and cell survival in neurons.

Given that human and rat VAP-A proteins share ~97% amino acid sequence identity, we investigated whether the defect in neurite outgrowth in PC12 cells depleted of VAP-A might be corrected by introduction of human VAP-A. Human VAP-A was resistant to shRNA-mediated interference because of a 4-nucleotide difference in the sequence corresponding to VAP-A-shRNA 2 between the human and rat mRNAs. Human VAP-A indeed restored the ability of NGF to induce neurite formation in PC12 cells that had been depleted of endogenous rat VAP-A (Fig. 8). Cell death induced by VAP-A depletion was also suppressed by expression of human VAP-A (data not shown). These results indicated that human VAP-A is functionally interchangeable with rat VAP-A.

We next tested the ability of VAP-A mutants to restore the induction of neurite outgrowth by NGF in PC12 cells depleted of endogenous VAP-A. Whereas the human VAP-A mutants K94D/M96D and ΔMSP, both of which interact with protrudin, restored NGF-induced neurite outgrowth to the same extent as did wild-type VAP-A, the mutant ΔTM, which does not reside in the ER and has lost the ability to bind to protrudin, failed to restore this effect of NGF (Fig. 8). These results suggested that the interaction of protrudin with VAP-A is likely indispensable for the neurite-extending function of protrudin. Alternatively, it remains possible that the failure of the ΔTM mutant to restore NGF-induced neurite formation in VAP-A-depleted PC12 cells might be explained by the transmembrane domain being essential for VAP-A localization and function rather than for interaction with protrudin.

VAP-A Is Required for Protrudin Localization—Protrudin resides in the ER of PC12 cells in the absence of NGF, whereas it is translocated to recycling endosomes in response to NGF stimulation (2). To investigate whether VAP-A might affect the subcellular distribution of protrudin in PC12 cells, we examined cells depleted of VAP-A or VAP-B by RNA interference (Fig. 9A). Immunofluorescence staining of control cells stimulated with NGF for 6 h revealed that endogenous protrudin was concentrated in the region containing recycling endosomes, whereas such transport of protrudin from the ER to recycling endosomes was not evident in cells depleted of VAP-A (Fig. 9B). Examination of VAP-A-depleted cells expressing FLAG-protrudin at longer times of exposure to NGF revealed that FLAG-protrudin became diffusely distributed throughout the cell body and that neurite extension was inhibited (Fig. 9C). In contrast, depletion of VAP-B affected neither the localization of protrudin nor neurite extension (Fig. 9D and data not shown). We examined the effect of VAP-A or VAP-B depletion (Fig. 9D) on the distribution of FLAG-protrudin in more detail in HeLa cells. Immunofluorescence corresponding to FLAG-protrudin was apparent only in the periphery of cells depleted of VAP-A, whereas it appeared to be localized to the ER in control cells and VAP-B-depleted cells (Fig. 9E). The localization of PDI was unaffected by VAP-A or VAP-B depletion. These results suggested that VAP-A is indispensable for ER retention of protrudin in HeLa cells, which is important for its neurite extending function.

DISCUSSION

Protrudin was discovered on the basis of its ability to induce process formation in non-neuronal cells, and this activity was shown to be essential for neurite outgrowth in neuronal cells (2, 5). Protrudin resides in the ER of neuronal cells in the absence of NGF, but it translocates to recycling endosomes in response to NGF stimulation and is ultimately transported to the tip of newly formed neurites (2). We have now investigated the molecular mechanism that underlies this translocation of protrudin by attempting to isolate proteins that interact with it and regulate its subcellular localization. Proteomics analysis led to...
Regulation of Protrudin Function by VAP

the identification of VAP-A and VAP-B as proteins that interact with protrudin. These proteins are thought to regulate the localization of proteins that contain an FFAT motif through interaction with this motif (11). VAP-A and VAP-B were thus candidates for regulators of protrudin localization. Indeed, we found that VAP-A interacts with protrudin through the FFAT motif of the latter and that it is required for the ER retention of protrudin in HeLa cells. In contrast, the NGF-induced transport of protrudin from the ER to recycling endosomes in PC12 cells was shown to be dependent on VAP-A, as was the process forming activity of protrudin. Although the reason for the difference in the effects of VAP-A depletion on protrudin localization between HeLa and PC12 cells (Fig. 9, B and E) remains unclear, it might be attributable to the difference in the extent of VAP-A depletion, which was partial in PC12 cells but almost complete in HeLa cells (Fig. 9, A and D) or to a difference in the inherent properties of the two cell types. Consistent with the latter notion, VAP-A depletion eventually results in cell death in PC12 cells but not in HeLa cells (data not shown). Depletion of VAP-A also triggers cell death in primary cultured rat hippocampal neurons (31).

The FFAT motif-containing proteins CERT (11), OSBP (25), and Nir-2 (12) interact with VAP and possess lipid binding activity. The interaction of VAP with the FFAT motifs of these proteins is required for their mediation of lipid transport between intracellular membrane systems. For example, in addition to an FFAT motif, CERT contains a Start domain, which interacts with ceramide and serves to mediate ER-Golgi trafficking of ceramide. Protrudin also contains a FYVE domain, which is thought to mediate the association of proteins with phosphatidylinositol 3-phosphate (32). These common characteristics of FFAT motif-containing proteins suggest that protrudin might contribute to vesicular transport through its association with lipids in the vesicular membrane and that this role might be regulated by VAP.

The MSP domain of VAP-A has been shown to be responsible for the binding of VAP-A to the FFAT motif of its target molecules. The crystal structure of a complex of VAP-A with the FFAT motif revealed that Lys<sup>87</sup> and Met<sup>89</sup> of rat VAP-A are important for the interaction (26). We have now shown that the corresponding residues of human VAP-A (Lys<sup>94</sup> and Met<sup>96</sup>) contribute to its interaction with protrudin. In contrast, mutation of other residues of human VAP-A corresponding to those implicated in the interaction of VAP-A with the FFAT motif, including Lys<sup>52</sup>, Thr<sup>53</sup>, Thr<sup>54</sup>, and Lys<sup>125</sup>, was found not to affect the binding of human VAP-A to protrudin. However, the fact that VAP and FFAT motif-containing proteins form a 2:2 tetramer (26) makes it difficult to interpret the results of such binding analyses in cells because of the presence of the endogenous wild-type protein. In addition to the MSP domain, the transmembrane domain of VAP-A also appeared to be important for the interaction with protrudin (Fig. 9F). The manner of the interaction between VAP-A and protrudin may thus differ somewhat from that of the interaction between VAP-A and other FFAT motif-containing proteins.

Dysfunction of VAP or protrudin is implicated in human neurological disorders. A mutation in the MSP domain (P56S) of human VAP-B has been associated with familial amyotrophic lateral sclerosis (33, 34). This condition is characterized by the death of motor neurons in the cerebral cortex, brainstem, and spinal cord, eventually resulting in muscle weakness and atrophy followed by death caused by respiratory failure 2–5 years after disease onset. The P56S mutation affects the interaction of VAP-B with other cellular proteins (35). ZFYVE27 (protrudin) was also found to be mutated in a German family with AD-HSP, which is characterized by the selective degeneration of axons (8). The phenotype of the affected individuals is similar to that of patients with AD-HSP caused by mutation of spastin, a protein implicated in the trafficking of vesicular cargo in neurons (9). Protrudin is thought to interact with spastin via its COOH-terminal FYVE domain (8). Further molecular analysis of VAP and protrudin may thus provide insight into the pathogenesis of these neurological disorders as well as a basis for the development of new therapeutic agents.

Acknowledgments—We thank S. Nagata for vectors; A. Miyawaki for Venus cDNA; M. Matsuoka for anti-VAP-B; A. Hamasaki, N. Nishimura, and other laboratory members for technical assistance; and A. Ohta and M. Kimura for help in preparation of the manuscript.

REFERENCES
Regulation of Protrudin Function by VAP

Promotion of Neurite Extension by Protrudin Requires Its Interaction with Vesicle-associated Membrane Protein-associated Protein
Shotaro Saita, Michiko Shirane, Tohru Natume, Shun-ichiro Iemura and Keiichi I. Nakayama

doi: 10.1074/jbc.M807938200 originally published online March 16, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M807938200

Alerts:
• When this article is cited  
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 14 of which can be accessed free at http://www.jbc.org/content/284/20/13766.full.html#ref-list-1