Roles of protrudin at interorganelle membrane contact sites

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Abstract: Intracellular organelles were long viewed as isolated compartments floating in the cytosol. However, this view has been radically changed within the last decade by the discovery that most organelles communicate with the endoplasmic reticulum (ER) network via membrane contact sites (MCSs) that are essential for intracellular homeostasis. Protrudin is an ER resident protein that was originally shown to regulate neurite formation by promoting endosome trafficking. More recently, however, protrudin has been found to serve as a tethering factor at MCSs. The roles performed by protrudin at MCSs are mediated by its various domains, including inactivation of the small GTPase Rab11, bending of the ER membrane, and functional interactions with other molecules such as the motor protein KIF5 and the ER protein VAP. Mutations in the protrudin gene (ZFYVE27) are associated with hereditary spastic paraplegia, an axonopathy that results from defective ER structure. This review, examines the pleiotropic molecular functions of protrudin and its role in interorganellar communication.

Keywords: protrudin, ZFYVE27, endoplasmic reticulum, membrane contact sites, organelle, trafficking

Introduction

The focus of research on intracellular organelles of eukaryotic cells has shifted dramatically in recent years from the characterization of each organelle compartment separately to the study of intercompartment communication. It is now widely accepted that organelles do not function independently but rather interact with the endoplasmic reticulum (ER) network via membrane contact sites (MCSs) throughout the cell. MCSs are microdomains where the membranes of the ER and other organelles come into close proximity (within a distance of <30 nm) in order to communicate with each other. However, the two membranes do not fuse, but rather maintain their separate identities.1–4) MCSs were first identified decades ago by classical electron microscopy, but the mechanisms underlying their formation and function remained unclear until recently. The development of advanced imaging technologies, such as three-dimensional, time-lapse, and high-resolution microscopy, as well as of fluorescent proteins for the visualization of MCSs, have made possible the recent progress in this area of research. MCSs have been shown to mediate lipid transfer, calcium ion regulation, and organelle dynamics such as endosome trafficking and mitochondrial fission (Fig. 1).5) Tethering factors that link the apposing membranes at MCSs have also recently been discovered and shown to play important roles in the formation and function of these structures.

Many organelles communicate with the ER via MCSs. The ER is a continuous membrane system that comprises the nuclear envelope, ribosome-
studded peripheral sheets (rough ER), and an interconnected tubular network (smooth ER). The structure of the smooth ER is supported by a group of membrane curvature proteins that contain a hydrophobic hairpin domain. Impaired function of these proteins results in the development of hereditary spastic paraplegia (HSP), a neurological disease caused by abnormal ER structure and function and the consequent evocation of ER stress and axonal degeneration.

Protrudin is a protein that plays a central role in directional endosomal trafficking by functioning as a tethering factor at MCSs.\(^6\),\(^7\) At MCSs formed between the ER and endosomes, protrudin promotes the transfer of endosomes from the ER to microtubules for their polarized transport. In addition, protrudin contributes to the regulation of ER structure. Mutations in the protrudin gene are also associated with HSP, suggesting that the integrity of endosome trafficking and ER structure regulated by protrudin is essential for the maintenance of neurons that extend long axons.

**Discovery and structural characteristics of protrudin**

Protrudin was first discovered as a protein of unknown function that binds to FK506 binding protein 38 (FKBP38), a multifunctional membrane chaperone that localizes to mitochondria and the ER.\(^8\)–\(^11\) In a yeast two-hybrid screen to select proteins that bind to FKBP38, we identified a novel protein that induced pronounced membrane deformity and the subsequent formation of long protrusions similar to neurites when it was overexpressed in cultured cells (Fig. 2). Thus, we named this protein “protrudin” on the basis of this activity.\(^6\) Since its discovery, we and others have uncovered molecular mechanisms underlying this process formation and other physiological functions of protrudin (Fig. 3).

Analysis of the predicted amino acid sequence of protrudin for functional domains revealed a Rab11 binding domain (RBD11), two transmembrane (TM) domains, a hairpin (HP) domain, a low complexity region (LCR), an two phenylalanines in an acidic tract (FFAT) motif, a coiled-coil domain, and a Fab1, YOTB, Vac1, EEA1 (FYVE) domain (Fig. 4). These structural characteristics provided clues to the functions of protrudin in the regulation of organelle dynamics such as directional endosome trafficking and ER morphogenesis.

**Protrudin regulates Rab11-dependent polarized trafficking**

Certain types of cell, such as epithelial cells and neurons, are polarized and possess distinct plasma membrane domains. Epithelial cells thus manifest apical and basolateral domains that face a lumen, such as the airway in the lungs, and the interior of the body, respectively. Similarly, neurons possess axonal and somatodendritic domains, which are specialized for the transmission of signals to and the reception of those from other cells, respectively. Cell polarity is largely dependent on recycling endosome traffic, or
transcytosis, regulated by the small GTPase Rab11. The active, GTP-bound form of Rab11 (Rab11-GTP) promotes directional trafficking from the apical to basolateral domains in epithelial cells as well as from the axonal to somatodendritic domains of neurons (Fig. 5).

As mentioned above, forced expression of protrudin in nonneuronal cells such as HeLa cells results in the generation of long processes with ruffling lamellipodia, a phenomenon that resembles neurite extension in neurons. Overexpression of protrudin was also found to promote axonal extension in hippocampal neurons. Conversely, depletion of protrudin in PC12 pheochromocytoma cells inhibited neurite outgrowth. In PC12 cells, neurite extension induced by nerve growth factor (NGF) is associated with sustained activation of extracellular signal-regulated kinase (ERK). This NGF-induced ERK activation results in the phosphorylation of protrudin, which in turn promotes its interaction with the inactive, GDP-bound form of Rab11 (Rab11-GDP). This binding promotes polarized endosome trafficking and consequent neurite outgrowth (Fig. 6).

The amino acid sequence of the RBD11 of protrudin is atypical in that the residues essential for interaction with Rab11-GTP are not conserved.

Fig. 3. (Color online) History of protrudin research. Major discoveries relating to protrudin are summarized. KO, knockout.

Fig. 4. (Color online) Domain structure of protrudin. Protrudin contains multiple functional domains. The Rab11 binding domain (RBD11) interacts with the GDP-bound form of Rab11 and thereby promotes polarized endosome trafficking. The amino acid sequence of protrudin RBD11 is similar to the corresponding sequence of guanine nucleotide dissociation inhibitors (GDIs). Protrudin resides at the ER, and its two transmembrane (TM) domains and a hairpin (HP) domain contribute to ER structure. The low complexity region (LCR) of protrudin interacts with the GTP-bound form of Rab7 and thereby promotes lysosome or late endosome trafficking. The two phenylalanines in an acidic tract (FFAT) motif of protrudin associates with the major sperm protein (MSP) domain of vesicle-associated membrane protein-associated protein (VAP) at MSCs. A sequence of seven amino acids (L) is specific to the L isoform of protrudin (see Fig. 11) and influences the binding affinity for VAP. A site near the coiled-coil (CC) domain of protrudin interacts with the kinesin protein KIF5 and thereby regulates microtubule-dependent endosome transport. Finally, the Fab1, YOTB, Vac1, EEA1 (FYVE) domain of protrudin associates with phosphatidylinositol phosphates (PIPs) and thereby mediates membrane tethering.
Rather, the sequence of RBD11 of protrudin is similar to the corresponding sequences of guanine nucleotide dissociation inhibitors (GDIs) that interact with the inactive, GDP-bound form of Rab proteins. Indeed, protrudin was shown to interact with a GTP binding-deficient mutant (S25N) of Rab11 that mimics Rab11-GDP and functions in a dominant negative manner, but not with a GTPase-deficient mutant (Q70L) that mimics Rab11-GTP and is constitutively active. Whereas Rab11-GTP promotes dendritic trafficking in neuronal cells, which was demonstrated by studies of translocation of the AMPA subtype of glutamate receptor to dendritic spines, Rab11-GDP inhibits such trafficking and consequently promotes axonal trafficking. The interaction of protrudin with Rab11-GDP stabilizes this inactive form of Rab11 and thus promotes axonal extension. We obtained further support for thus scenario with the observation that the axons of neurons are shortened in mice deficient in protrudin.

In addition to binding to Rab11 to regulate recycling endosome trafficking, protrudin interacts with Rab7 and thereby regulates late endosome and lysosome trafficking. However, binding to Rab7 is mediated by the LCR in the central region of protrudin rather than by RBD11 in the NH2-terminal region. In addition, protrudin binds to the GTP-bound form of Rab7, in contrast to its binding to the GDP-bound form of Rab11. These findings suggest that protrudin independently regulates different types of organelle transport in a context-dependent manner through interaction with various Rab proteins.

**Mutation of protrudin impairs ER structural integrity and gives rise to HSP**

Protrudin is an ER-resident membrane protein that is preferentially localized to the tubular ER (Fig. 7). Protrudin promotes the fusion of ER tubules and ER network formation, and it has been shown to increase the density of three-way junctions especially at the cell periphery. Depletion of protrudin by RNA interference in HeLa cells renders the sheet-like structure of the ER more prominent, suggesting that protrudin contributes to regulation of the sheet-versus-tubule balance in the structure of this organelle.

The initial identification of three hydrophobic regions thought to serve as TM domains in protrudin proved to be inconsistent with the experimentally determined topology of the protein, in which both the NH2-terminal RBD11 and the COOH-terminal FYVE domain are located in the cytosol. It was subsequently realized that the putative third TM domain, now known as the HP domain, does not
actually penetrate the membrane but rather is inserted into the lipid bilayer only on the cytosolic side. This one-sided insertion of the HP domain bends the ER membrane and thereby increases its curvature.\(^\text{12}\)

The protrudin gene (ZFYVE27) is mutated in a subset of individuals with HSP, and protrudin is now referred to as spastic paraplegia (SPG) 33.\(^\text{13}\)

HSP is characterized by selective degeneration of long axons that connect upper cortical neurons, which gives rise to spastic gait disorders (Fig. 8). In addition to the original German family of an HSP patient found to have a mutation (G191V) in the HP domain of protrudin, an Australian family with a mutation (D284G) adjacent to the FFAT motif has since been identified (Fig. 8). Proteomics analysis of the brain of neuron-specific protrudin transgenic mice revealed that protrudin interacts in vivo with multiple HSP-related proteins including myelin proteolipid protein 1 (SPG2), atlastin-1 (SPG3A),
REEP1 (SPG31), REEP5, KIF5A (SPG10), KIF5B, KIF5C, and reticulons 1, 3, and 4 (which are similar to reticulon 2, also known as SPG12). Most cases of HSP result from autosomal dominant mutations in the genes for atlastin-1, REEP1, spastin (SPG4), and reticulon proteins. Like protrudin, all of these proteins harbor a hydrophobic HP domain that shapes high-curvature ER tubules (Fig. 9). Mutations in this domain give rise to an abnormal ER morphology and increased susceptibility to ER stress, a major contributor to HSP pathogenesis. Indeed, forced expression of the G191V mutant of protrudin, but not that of the wild-type protein, evokes ER stress in cultured cells. Although this effect of the mutant protein is relatively modest, the ER stress likely accumulates over a long period of time (decades) before the onset of symptoms in individuals with HSP. Some SPG proteins have been functionally linked to ER-associated degradation (ERAD), a multistep pathway encompassing the degradation of ER proteins by the ubiquitin-proteasome system. The half-life of the protrudin(G191V) mutant in Neuro2A cells is markedly longer than that of the wild-type protein, suggesting that the HSP-associated mutation of protrudin may result in a defect in the ERAD system. It is possible that protrudin(G191V) is misfolded in the ER, which results in a defect in the ERAD system that enhances the ER stress response and thereby promotes the pathogenesis of HSP.

VAP is essential for ER localization and the neurite-extending function of protrudin

Our proteomics analysis of proteins that bind to protrudin also identified several molecules that account for key properties of protrudin including vesicle-associated membrane protein-associated protein (VAP) and the kinesin protein KIF5. Its interaction with the ER resident protein VAP is consistent with the presence of the FFAT motif in protrudin, given that VAP contains a major sperm protein (MSP) domain that interacts with this motif. Both the interaction of protrudin with VAP and the induction of process formation by protrudin were found to be markedly inhibited by mutation of the FFAT motif. Furthermore, depletion of VAP by RNA interference resulted in mislocalization of protrudin as well as in inhibition of NGF-induced neurite outgrowth in PC12 cells. These observations suggested that VAP is indispensable for the ER retention of protrudin, which is in turn important for its neurite-extending function.

Protrudin regulates KIF5-dependent endosome trafficking at MCSs

The identification of KIF5 as a protrudin binding protein provided insight into the mechanism by which protrudin induces polarized membrane trafficking, given that KIF5 is a microtubule-dependent motor protein that mediates anterогrade cargo trafficking in axons. Endosomes that are tethered to the ER at MCSs by protrudin are charged with KIF5 and released onto microtubules for transport mediated by KIF5 toward the plus-end of the tubules. This process of endosome tethering and release is promoted by protrudin and is repeated to give rise to a “ping-pong” movement of endosomes that drives polarized endosome traffic to the cell.
Protrudin binds to the heavy chain of KIF5 and transfers it to the late endosomal protein FYCO1, which is a motor adaptor and also binds to the light chain of KIF5. Loading with KIF5 is required for the movement of endosomes to the cell periphery, where they can undergo synaptotagmin VII-dependent fusion with the plasma membrane, which supports the formation of neurites or other cellular protrusions. Protrudin binds to the heavy chain of KIF5 and transfers it to the late endosomal protein FYCO1, which is a motor adaptor and also binds to the light chain of KIF5. Loading with KIF5 is required for the movement of endosomes to the cell periphery, where they can undergo synaptotagmin VII-dependent fusion with the plasma membrane, which supports the formation of neurites or other cellular protrusions.

**The FYVE domain of protrudin facilitates membrane fusion at the plasma membrane**

Proteins that contain an FYVE domain have been thought to contribute to organelle dynamics by serving as tethering factors for membrane fusion and thereby promoting the formation of SNARE complexes. The typical FYVE domain is a zinc-containing module of 60–80 amino acids and possesses the highly basic sequence motif (R/K)(R/K)HHCR. This conserved motif is essential for binding to phosphatidylinositol 3-phosphate (PI(3)P), which is present predominantly in the membrane of endosomes. Three-dimensional structural analysis of the FYVE domains of Vps27p or EEA1 bound to PI(3)P has revealed that the negatively charged 3-phosphate group of PI(3)P interacts tightly with positively charged amino acids in the (R/K)(R/K)HHCR motif of the FYVE domain. However, these basic residues are not conserved in the FYVE domain of protrudin, which is unique in terms of its lipid binding properties and subcellular localization. Surface plasmon resonance analysis showed that the FYVE domain of protrudin interacts with multiple phosphatidylinositol phosphates (PIPs) including PI(4,5)P2, PI(3,4)P2, and PI(3,4,5)P3. In addition, we found that this domain interacts with multiple PIPs including PI(3)P, PI(4)P, and PI(5)P by liposome binding analysis and an overlay assay. A fluorescent protein fused to the FYVE domain of protrudin was also shown to localize to the plasma membrane and to promote the fusion of endosomes to the plasma membrane in cultured cells. These characteristics of the FYVE domain of protrudin are key to the ability of protrudin to promote vesicle trafficking that underlies neurite outgrowth.

**Neuron-specific splicing isoform of protrudin**

Protrudin precursor mRNA is alternatively spliced to generate mature transcripts for two different isoforms of protrudin, designated L and S (for long and short). Protrudin-S appears to be expressed in all tissues, whereas protrudin-L is expressed specifically in the nervous system. Protrudin-L contains an additional seven amino acids (encoded
by exon L) compared with protrudin-S. The sequence encoded by exon L is located adjacent to the FFAT motif, which binds to VAP, with the result that protrudin-L binds to VAP with higher affinity than protrudin-S. This difference in binding affinity likely accounts for the observation that protrudin-L is more effective at promoting neurite outgrowth than protrudin-S. A neural-specific splicing regulator, SRRM4, which is required for neurogenesis, promotes the splicing of protrudin pre-mRNA to yield protrudin-L mRNA (Fig. 11).25) Mutations of the SRRM4 gene have been associated with psychiatric disorders, suggesting the possibility that protrudin may also be related to the pathogenesis of such disorders.

**Perspective**

The interorganelle network established by MCSs plays a key role in cell homeostasis, with MCSs contributing to lipid metabolism, calcium ion regulation, and organelle dynamics. Evidence now indicates that protrudin is essential for the function of MCSs, in particular with regard to endosome trafficking. The recent rapid progress in protrudin research relating to MCSs suggests that additional roles for these structures may remain to be uncovered. Studies with protrudin-deficient mice in particular may help to reveal such roles.

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**References**

1) Helle, S.C., Kanfer, G., Kolar, K., Lang, A., Michel, A.H. and Kornmann, B. (2013) Organization and function of membrane contact sites. Biochim. Biophys. Acta 1833, 2526–2541.
2) Phillips, M.J. and Voeltz, G.K. (2016) Structure and function of ER membrane contact sites with other organelles. Nat. Rev. Mol. Cell Biol. 17, 69–82.
3) Salvador-Gallego, R., Hoyer, M.J. and Voeltz, G.K. (2017) SnapShot: Functions of endoplasmic reticulum membrane contact sites. Cell 171, 1224–1224.e1.
4) Wu, H., Carvalho, P. and Voeltz, G.K. (2018) Here, there, and everywhere: The importance of ER membrane contact sites. Science 361, eaan5835.
5) Friedman, J.R., Dibenedetto, J.R., West, M., Rowland, A.A. and Voeltz, G.K. (2013) Endoplasmic reticulum-endosome contact increases as endosomes traffic and mature. Mol. Biol. Cell 24, 1030–1040.
6) Shirane, M. and Nakayama, K.I. (2006) Protrudin induces neurite formation by directional membrane trafficking. Science 314, 818–821.
7) Raiborg, C., Wenzel, E.M., Pedersen, N.M., Olsvik, H., Schink, K.O., Schultz, S.W. et al. (2015) Repeated ER-endosome contacts promote endosome translocation and neurite outgrowth. Nature 520, 234–238.
8) Shirane, M. and Nakayama, K.I. (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat. Cell Biol. 5, 28–37.
9) Shirane, M., Ogawa, M., Motoyama, J. and Nakayama, K.I. (2008) Regulation of apoptosis

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Fig. 11. (Color online) Molecular structure of neuron-specific splicing isoform protrudin-L. Protrudin pre-mRNA is alternatively spliced to generate protrudin-L and protrudin-S mRNAs. Protrudin-L is a neuron-specific isoform that contains an additional seven amino acids (encoded by exon L) compared with protrudin-S. The sequence encoded by exon L is located adjacent to the FFAT motif that binds to VAP, with the result that protrudin-L binds to VAP with higher affinity than protrudin-S. The neural-specific splicing regulator SRRM4 promotes the splicing of protrudin pre-mRNA to produce protrudin-L mRNA.
and neurite extension by FKBP38 is required for neural tube formation in the mouse. Genes Cells 13, 635–651.

10) Saita, S., Shirane, M. and Nakayama, K.I. (2013) Selective escape of proteins from the mitochondria during mitophagy. Nat. Commun. 4, 1410.

11) Shirane-Kitsuji, M. and Nakayama, K.I. (2014) Mitochondria: FKBP38 and mitochondrial degradation. Int. J. Biochem. Cell Biol. 51, 19–22.

12) Hashimoto, Y., Shirane, M., Matsuzaki, F., Saita, S., Ohnishi, T. and Nakayama, K.I. (2014) Protrudin regulates endoplasmic reticulum morphology and function associated with the pathogenesis of hereditary spastic paraplegia. J. Biol. Chem. 289, 12946–12961.

13) Mannan, A.U., Krawen, P., Sauter, S.M., Boehm, J., Chronowska, A., Paulus, W. et al. (2006) ZFYVE27 (SPG33), a novel spastin-binding protein, is mutated in hereditary spastic paraplegia. Am. J. Hum. Genet. 79, 351–357.

14) Blackstone, C. (2012) Cellular pathways of hereditary spastic paraplegia. Annu. Rev. Neurosci. 35, 25–47.

15) Hubner, C.A. and Kurth, I. (2014) Membrane-shaping disorders: A common pathway in axon degeneration. Brain 137, 3109–3121.

16) Zhang, C., Li, D., Ma, Y., Yan, J., Yang, B., Li, P. et al. (2012) Role of spastin and protrudin in neurite outgrowth. J. Cell. Biochem. 113, 2296–2307.

17) Chang, J., Lee, S. and Blackstone, C. (2013) Protrudin binds atlastins and endoplasmic reticulum-shaping proteins and regulates network formation. Proc. Natl. Acad. Sci. U.S.A. 110, 14954–14959.

18) Saita, S., Shirane, M., Natsume, T., Jenura, S. and Nakayama, K.I. (2009) Promotion of neurite extension by protrudin requires its interaction with vesicle-associated membrane protein-associated protein. J. Biol. Chem. 284, 13766–13777.

19) Matsuzaki, F., Shirane, M., Matsumoto, M. and Nakayama, K.I. (2011) Protrudin serves as an adaptor molecule that connects KIF5 and its cargoes in vesicular transport during process formation. Mol. Biol. Cell 22, 4602–4620.

20) Krauss, M. and Haucke, V. (2015) A grab to move on: ER-endosome contacts in membrane protrusion formation and neurite outgrowth. EMBO J. 34, 1442–1444.

21) Raiborg, C., Wenzel, E.M. and Stenmark, H. (2015) ER-endosome contact sites: Molecular compositions and functions. EMBO J. 34, 1848–1858.

22) Wijdeven, R.H., Jongsma, M.L., Neefjes, J. and Berlin, I. (2015) ER contact sites direct late endosome transport. BioEssays 37, 1298–1302.

23) Gil, J.E., Kim, E., Kim, I.S., Ku, B., Park, W.S., Oh, B.H. et al. (2012) Phosphoinositides differentially regulate protrudin localization through the FYVE domain. J. Biol. Chem. 287, 41268–41276.

24) Ohnishi, T., Shirane, M., Hashimoto, Y., Saita, S. and Nakayama, K.I. (2014) Identification and characterization of a neuron-specific isoform of protrudin. Genes Cells 19, 97–111.

25) Ohnishi, T., Shirane, M. and Nakayama, K.I. (2017) SRRM4-dependent neuron-specific alternative splicing of protrudin transcripts regulates neurite outgrowth. Sci. Rep. 7, 41130.

Profile

Michiko Shirane was born in Hikone city, Shiga prefecture in 1967. She graduated from Osaka University School of Science in 1990 and received a PhD degree at the University of Tokyo in 1999. She worked as a researcher on a Research Fellowship for Young Scientists of Japan Society for the Promotion of Science (JSPS) from 2000 and Precursory Research for Embryonic Science and Technology (PRESTO) from 2003, and became an Assistant Professor in 2003 and an Associate Professor in 2007 at Kyushu University under Prof. K. I. Nakayama. She became a Professor at Nagoya City University Graduate School of Pharmaceutical Sciences in 2017. She discovered the protein protrudin, which is a key regulator for intracellular trafficking in neuronal cells, and published its function in Science in 2006. She has subsequently been investigating organelle dynamics associated with protrudin including membrane contact sites. For her accomplishments, she received the JSPS Prize in 2009.