Characterization of Amyotrophic Lateral Sclerosis-linked P56S Mutation of Vesicle-associated Membrane Protein-associated Protein B (VAPB/ALS8)*

Received for publication, May 26, 2006, and in revised form, July 28, 2006. Published, JBC Papers in Press, August 4, 2006, DOI 10.1074/jbc.M605049200

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The P56S mutation in VAPB (vesicle-associated membrane protein-associated protein B) causes autosomal dominant motoneuronal diseases. Although it was reported that the P56S mutation induces localization shift of VAPB from endoplasmic reticulum (ER) to non-ER compartments, it remains unclear what the physiological function of VAPB is and how the P56S mutation in VAPB causes motoneuronal diseases. Here we demonstrate that overexpression of wild type VAPB (wt-VAPB) promotes unfolded protein response (UPR), which is an ER reaction to suppress accumulation of misfolded proteins, and that small interfering RNA for VAPB attenuates UPR to chemically induced ER stresses, indicating that VAPB is physiologically involved in UPR. The P56S mutation nullifies the function of VAPB to mediate UPR by inhibiting folding of VAPB that results in insolubility and aggregate formation of VAPB in non-ER fractions. Furthermore, we have found that expression of P56S-VAPB inhibits UPR, mediated by endogenous wt-VAPB, by inducing aggregate formation and mislocalization into non-ER fractions of wt-VAPB. Consequently, the P56S mutation in a single allele of the VAPB gene may diminish the activity of VAPB to mediate UPR to less than half the normal level. We thus speculate that the malfunction of VAPB to mediate UPR, caused by the P56S mutation, may contribute to the development of motoneuronal degeneration linked to VAPB/ALS8.

Amyotrophic lateral sclerosis (ALS) is the most prevalent fatal motor neuron disease, characterized by progressive loss of upper and lower motor neurons (1, 2). Although typical cases occur sporadically, some patients have a genetic background.

To date, four ALS-causative genes have been identified, and precise characterization of their physiological roles and abnormalities by ALS-causing mutations is bringing us clues as to how ALS and other motor neuron diseases occur. Overexpression of mutants of Cu/Zn-superoxide dismutase (SOD1), whose gene is the most characterized familial ALS-related one known as ALS1, causes neuronal cell death in vitro (3, 4) and an ALS-like phenotype in vivo (5, 6). A recently identified autosomal recessive ALS-causative gene, ALS2, encodes alsin protein (7, 8) that has several functional domains common to Rho guanine nucleotide-exchanging factors (RhoGEF) (9) and Rab5GFE (10). We have recently demonstrated that alsin exerts neuroprotective function via its RhoGEF domain against neurotoxicity by SOD1 mutants in vitro (4). Ablation of the ALS2 gene caused mild motor disorder (11). ALS4, a recently identified autosomal dominant ALS-associated gene, is thought to encode a DNA/RNA helicase whose function remains unknown (12).

ALS8, encoding mutated VAPB (vesicle-associated membrane protein-associated protein B), was most recently identified from a large Brazilian family with autosomal dominant motor neuron diseases. The P56S point mutation in VAPB caused a typical ALS phenotype with rapid progression or late onset spinal muscular atrophy (SMA) (13). This mutation has affected eight families totaling 1500 individuals, of whom 200 suffer from ALS/SMA (14).

The human VAP family proteins were initially identified in Aplysia californica (aVAP33) (15) that is involved in exocytosis of neurotransmitters (16). They include VAPA (also known as VAP33), VAPB, and VAPC. VAPB and VAPC are alternatively spliced variants, VAPA and VAPB, which interact with each other, associate with VAMP/synaptobrevin (15). It was subsequently demonstrated that the yeast VAP homologue, called SCS2 (suppressor of choline sensitivity 2), compensates for the defect of Ire1 and Hac1 (17). Ire1 and Hac1 encode yeast homologues of mammalian Ire1 (inositol-requiring enzyme-1) and mammalian transcriptional factor XBP1 (X-box-binding protein-1). Ire1 and XBP1 play a pivotal role in inositol metabolism and unfolded protein response (UPR) (18), an ER process to suppress accumulation of unfolded protein in ER (19). Several other reports

* This work was supported by a grant from the Japan Society for the Promotion of Science Research Fellow. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: ALS, amyotrophic lateral sclerosis; SMA, spinal muscular atrophy; VAMP, vesicle-associated membrane protein; VAP, VAMP-associated protein; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; UPR, unfolded protein response; TMD, transmembrane domain; GST, glutathione S-transferase; ER, endoplasmic reticulum; wt-VAPB, wild type VAPB; HRP, horseradish peroxidase; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid; siVAPB, small interfering RNA for VAPB; MBS, MES-buffered saline.
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have also suggested that VAPB may play important roles in the structural regulation of ER, protein transport, phospholipid metabolism, and also viral infection (17, 20, 21).

Based on the foregoing studies, it is speculated that VAPB is involved in ER function, especially UPR induction mediated by the IRE1/XBP1 pathway, and in inositol metabolism (22, 23), although there is no direct evidence supporting this idea. Moreover, it remains unclear how the ALS-causing P56S-VAPB mutant participates in the pathomechanism of ALS. So far, the sole reported finding relating to the latter issue is that P56S-VAPB localizes in non-ER and non-Golgi compartments, whereas wt-VAPB does so in ER and the Golgi apparatus (13).

In this study, we demonstrate that VAPB plays an important role in UPR. We further show that the P56S mutation causes almost complete loss of function of VAPB to mediate UPR by inducing its misfolding and localization shift to the non-ER compartments. In addition, P56S-VAPB suppresses UPR, mediated by wt-VAPB, by interfering with the folding of wt-VAPB. Inhibition of wt-VAPB-mediated UPR by co-expressed P56S-VAPB may occur possibly because wt-VAPB is firmly dimerized with P56S-VAPB and may be trapped into inactive homodimerized protein complexes. Considering the data together, we speculate that the function of VAPB to mediate UPR may be diminished to less than half of the normal level by the P56S mutation in a single allele of the VAPB gene. We further speculate that the malfunction of VAPB to mediate UPR, induced by the P56S mutation, may eventually contribute to the development of motoneuronal degeneration linked to ALS by permitting the accumulation of misfolded proteins in the ER.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Compounds*—Rabbit anti-VAPB polyclonal antibody was purchased from Abcam (Cambridge, UK). Immunoblot analysis with this antibody only detects overexpression levels of VAPB, but not endogenous levels of VAPB, in NSC34 cells. Rabbit polyclonal anti-VAPB-P antibody was raised against an N-terminal human VAPB peptide (N-terminal 14 residues) by Tanpaku Seisei Kogyo. Anti-FLAG M2 monoclonal antibody, horseradish peroxidase (HRP)-conjugated anti-FLAG M2 monoclonal antibody, rabbit anti-actin polyclonal antibody were purchased from Bio-Rad (Hercules, CA). Rabbit polyclonal anti-calreticulin antibody and anti-calnexin antibody were purchased from Stressgen (Victoria, Canada). Texas Red-conjugated anti-rabbit polyclonal antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The following mouse monoclonal antibodies were purchased from companies: anti-Myc antibody, Biomol (Plymouth Meeting, PA); HRP-conjugated anti-HA antibody, Roche Applied Science; anti-GST-antibody, Upstate (Charlottesville, VA); anti-tubulin antibody, Oncogene (Cambridge, MA). Thapsigargin, bafilomycin A, and brefeldin A were purchased from Sigma. MG132 was purchased from Calbiochem.

**Constructions**—Human cDNAs encoding VAPB (GenBank access number NM_004738), VAPA (GenBank access number BT019618), VAMP1 (GenBank access number NM_014231), and VAMP2 (GenBank access number NM_014232) were amplified from a human postcentral gyrus cDNA library (Biochain, Hayward, CA) by PCR with a sense primer (5′-CGGGATCCAACATGGAAGTGAGACGGGACGAGCAGTC-3′) and an antisense primer (5′-GGAATCTCACAGGCAATCTCTTCAATAATTAC-3′) for human VAPB, a sense primer (5′-CGGGATCCATTGGAAGTGAGACGGGACGAGCAGTC-3′) and an antisense primer (5′-GGAATTTCTCACAGGCAATCTCTTCAATAATTAC-3′) for VAPA, a sense primer (5′-CGGGATCCACATGGAAGTGAGACGGGACGAGCAGTC-3′) and an antisense primer (5′-GGAATTTCTCACAGGCAATCTCTTCAATAATTAC-3′) for VAMP1, and a sense primer (5′-CGGGATCCACATGGAAGTGAGACGGGACGAGCAGTC-3′) and an antisense primer (5′-GGAATTTCTCACAGGCAATCTCTTCAATAATTAC-3′) for VAMP2, respectively.

P56S-VAPB, P56S-VAPA, P56A-VAPB, P56K-VAPB, P56D-VAPB, P56del-VAPB, and K87D/M89D-VAPB were obtained by site-directed mutagenesis with a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for P56S-VAPB and P56S-VAPA, a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for P56A-VAPB, a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for P56K-VAPB, a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for P56D-VAPB, a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for P56del-VAPB, and a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for VAMP1, and a sense primer (5′-GGTACTGTTGAGGTCACACAGGGAATCATCAG-3′) and an antisense primer (5′-CGATGATTCTGGCCTGAGCTCACACGTACC-3′) for VAMP2, respectively.

Two oligonucleotides, a sense fragment (5′-TCTTCTACACAGTACC-3′) and an antisense fragment (5′-CCCAATGAGAAAAGTAAAACACGACTTTGACTGTTGCCCCCTACACAGTACC-3′) were annealed in vitro and subcloned into the BamH1-KpnI site of plasmid DNA for silencing of endogenous VAPB was constructed as follows. Two oligonucleotides, a sense fragment (5′- CGGGA-CTCCGTAGACTGAAACTACATTTTGTGAGCTT- CCTCTACACAGTACC-3′) and an antisense fragment (5′-GGAATTTCTTCAAAATGACTGAACTACATTTTGTGAGCTT- CCTCTACACAGTACC-3′) were annealed in vitro and subcloned into the BamH1-KpnI site of pRNA-U6.1/Shuttle vector (Genscript). pcX-F-XBP1-Venus and pcAX-F-XBP1-DDBD-Venus constructs were kindly provided by Dr. Masayuki Miura (Tokyo University). pXJ-HA-ubiquitin plasmid was a kind gift from Dr. Victor Yu (National University of Singapore).
Cell Culture and Transfection—Motoneuronal NSC34 cells, a hybrid cell line established from a mouse neuroblastoma cell line and mouse embryo spinal cord cells, was a kind gift from Dr. Neil Cashman (Toronto University). NSC34 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% of fetal bovine serum (HyClone, Logan, UT). NSC34 cells were seeded onto a 6-cm culture dish at 2.1 × 10⁶ cells/dish 24 h before transfection. Transfection was performed via their C-terminal TMDs.

VAPB contains three conserved domains: the N-terminal major sperm protein domain, the middle coiled-coil domain, and the C-terminal transmembrane domain.

Immunoblot Analysis—Samples were mixed with equal amounts of 2× sample buffer containing 4% SDS and boiled for 5 min at 95 °C. The samples were applied to SDS-polyacrylamide gels and blotted onto polyvinylidene fluoride membranes. Immunoreactive bands were detected with ECL Western blotting detection reagents (Amersham Biosciences). Intensities of immunodetected signals were densitometrically estimated with NIH Image.

Immunocytochemistry—COS7 cells, plated onto cell culture dishes, were transfected with N-terminally EGFP-tagged VAPB by lipofection with Lipofectamine and PLUS reagent. Forty-eight h after transfection, the cells were fixed with –3% paraformaldehyde plus phosphate-buffered saline. ER was probed by incubation with an anti-calreticulin antibody (Stressgen) and visualized by Texas Red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories). The cells were observed by confocal microscopy LSM510 (Carl Zeiss).

Pull-down Assay—NSC34 cells, transiently expressing GST-fused proteins and His₆-Xpress-tagged proteins, were harvested 48 h after transfection and lysed with a pull-down buffer (150 mM NaCl, 20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Triton X-100, protease inhibitors) by pipetting and a freeze-thaw cycle. After centrifugation, the cell lysates were precleared with Sepharose beads for 4 h and pulled down with glutathione-Sepharose beads (Amersham Biosciences) for 4 h. After being washed four times with the pull-down buffer, the precipitates were immunoblotted with anti-HisG antibody (for the detection of His₆-Xpress-tagged proteins) and anti-GST antibody.

Ubiquitination Assay—NSC34 cells, cotransfected with the vector encoding HA-ubiquitin in association with the pEF4/His vector, pEF4/His-wt-VAPB, or pEF4/His-P56S-VAPB, were harvested 48 h after transfection for lysis with the radioimmune precipitation buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). The cell lysates were then precleared with Sepharose beads for 4 h and immunoprecipitated with anti-Xpress antibody. After being washed four times with the radioimmune precipitation buffer, the precipitates were immunoblotted with HRP-conjugated anti-HA antibody (to detect ubiquitinated proteins) and anti-HisG antibody (to detect VAPB).

Fractionation into Soluble and Insoluble Fractions—NSC34 cells overexpressing VAPB were harvested 48 h after transfection for lysis with a cell lysis buffer (10 mM Tris–HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, protease inhibitors) by pipetting and a freeze-thaw cycle. The soluble fraction was defined as the supernatant of the cell lysates after centrifugation at 12,000 × g for 5 min. After complete removal of the supernatant, the pellets were resuspended in 500 µl of the cell lysis buffer and sonicated for 10 s to homogenize the mixture. The solutions were then centrifuged for 5 min at 12,000 × g, and the supernatant was completely removed. The resulting pellets, defined as the insoluble fractions, were solubilized by pipetting in the 4% SDS-containing sample buffer for subsequent immunoblot analysis.

Fractionation by Sucrose Density Gradient Centrifugation—Untransfected NSC34 cells or NSC34 cells transfected with VAPB-encoding vectors were harvested for suspension in the 1% Triton X-100-MBS lysis buffer (1% Triton-X100, 25 mM MES (pH 6.7), 150 mM NaCl) and rotated for 20 min at 4 °C, followed by 10 passages through 26-gauge needles. The suspended total cell lysates, mixed with an equal volume of 80% sucrose plus MBS (final 40% sucrose), were initially loaded to 4.5-ml ultracentrifuge tubes (Beckman). A discontinuous
sucrose gradient was then formed by sequentially layering 30% sucrose-MBS and 5% sucrose plus MBS. After the tubes were subjected to ultracentrifugation at 260,000 \( \times g \) for 18 h in Beckman SW-Ti60 rotor at 4 °C, the gradient was divided into the same volume (350 \( \mu l \)) of fractions from the top to the bottom of the tube. The pellet was sonicated to suspend in 350 \( \mu l \) of the 1% Triton X-100-MBS lysis buffer. An equal volume (20 \( \mu l \)) of each fraction was then analyzed by Western blot.

RESULTS

The P56S Mutation Induces the Insolubility of VAPB—We cloned a human wt-VAPB cDNA, from which the P56S-VAPB mutant cDNA was generated by site-directed mutagenesis (Fig. 1A). The 56th proline, located at the middle of the major sperm protein domain, is highly conserved among VAP family proteins derived from various species. We constructed their expression vectors to express various epitope-tagged proteins (Fig. 1B).

It has been generally accepted that many neurodegenerative disease-linked mutations result in misfolding and aggregation of disease-related proteins (24–26). Misfolded proteins are prone to be easily polyubiquitinated (27, 28). To assess whether the P56S mutation induces misfolding and aggregation of VAPB, we first examined the status of polyubiquitination of P56S-VAPB. As shown in Fig. 2A, P56S-VAPB was more ubiquitinated than wt-VAPB, supporting the idea that P56S-VAPB was also misfolded. Note that the expression level of P56S-VAPB was lower than that of wt-VAPB, which might be due to the change in solubility. Figure 2B shows that the P56S mutation increased the Triton X-100-insoluble fraction of VAPB protein. Figure 2C–G demonstrates that the P56S mutation increased the fraction of VAPB that remained in the ER, as shown by the increased distance from the ER membrane marker calnexin.
VAPB is much lower than that of wt-VAPB despite transfection with same amount of plasmids.

Based on these findings, we tested whether the P56S mutation increased insolubility of VAPB in the Triton X-100-containing cell lysis buffer (1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, protease inhibitors). As shown in Fig. 2B, the amount of the Triton X-100-soluble P56S-VAPB was much smaller than that of Triton X-100-soluble wt-VAPB, whereas the amount of the Triton X-100-insoluble P56S-VAPB was much greater than the Triton X-100-insoluble wt-VAPB. As expected, a smeared ladder, presumably composed of super-shifted P56S-VAPB proteins, was detected in the insoluble fraction of P56S-VAPB. These higher molecular weight bands were assumed to be SDS-containing buffer-insoluble P56S-VAPB with some modifications or P56S-VAPB aggregates. Thus, it was concluded that the P56S mutation reduced the solubility of VAPB proteins by inducing misfolding of VAPB protein. Considering that VAPB is homodimerized or heterodimerized with other synaptic proteins via the C-terminal transmembrane domain (TMD) (15, 21) and that the P56S mutation does not interfere with this oligomerization (data not shown, but see Fig. 6), VAPB-interacting proteins may be trapped into insoluble aggregates of P56S-VAPB and contribute to the VAPB-containing smeared ladder.

**P56S-VAPB Shows Different Subcellular Localization**—wt-VAPB has been known to mainly localize in ER (13, 20, 21). It has been also demonstrated that the P56S-VAPB mutant does not co-localize with the Golgi apparatus or ER (13). To visualize these proteins in situ, we constructed enhanced green fluorescent protein (EGFP)-tagged VAPB. As shown in Fig. 2C, EGFP-wt-VAPB showed reticulated structures co-localizing with calreticulin, an ER marker. On the other hand, P56S-VAPB that showed fine dotlike structures did not co-localize with ER (Fig. 2D).

It has been known that some synaptic vesicular proteins, such as VAMP2, are known to localize in a scattered membrane fraction, called “raft” (30). Raft is known to be insoluble in the Triton X-100-containing buffer. Taken altogether, it is possible that a minor portion of wt-VAPB, a putative synaptic vesicular protein, may localize in the membrane raft and that the P56S mutation may enhance the localization of VAPB in the membrane raft. To examine the possibility that the P56S mutation abnormally enhances the localization of VAPB into the membrane raft and to confirm the subcellular localization pattern of wt-VAPB and P56S-VAPB, we fractionated these proteins by sucrose density gradient centrifugation in the Triton X-100-containing buffer (30).

For this purpose, untransfected NSC34 cells or NSC34 cells transfected with pEF4/His-wt-VAPB or pEF4/His-P56S-VAPB were harvested for suspension and homogenization in the Triton X-100-MBS buffer, and the total lysates were then subjected to sucrose density gradient centrifugation. As shown in Fig. 2E, most endogenous wt-VAPB was distributed in 40% sucrose fractions. The fractionation pattern of endogenous wt-VAPB was almost equal to that of calnexin, an ER membrane protein, and calreticulin, a soluble ER protein (Fig. 2E, bottom). Overexpressed wt-VAPB showed the same fractionation pattern as endogenous wt-VAPB (Fig. 2F). Similar to other synaptic vesicular proteins, such as VAMP2, a minor portion of wt-VAPB was distributed in several consecutive fractions around the border between 5 and 30% sucrose, suggesting that the minority of VAPB localizes in the Triton X-100-insoluble membrane raft fraction. In contrast, a major part of P56S-VAPB was fractionated into the Triton X-100-insoluble pellet (Fig. 2G) but not into the Triton X-100-insoluble raft, indicating that the P56S mutation induced the localization shift of VAPB into the non-ER and non-raft Triton X-100-insoluble subcellular compartments.

**Overexpression of wt-VAPB, but Not That of P56S-VAPB, Induces UPR**—Several groups have predicted the involvement of mammalian VAP as well as SCS2 in UPR, in addition to inositol metabolism (22, 23). Based on these predictions, we hypothesized that VAPB may be involved in UPR and that the P56S mutation may affect it.

In this study, to monitor UPR, we used a tool developed by Iwawaki et al. (29) (Fig. 3). Under normal conditions, mRNA of XBP1 is spliced by activated IRE1 in UPR. Messenger RNA of XBP1 is spliced by activated IRE1 in UPR. Under normal conditions, splicing of XBP1 mRNA by IRE1 does not occur, and immature XBP1 protein is produced. Under ER stress conditions, splicing by IRE1 occurs, followed by expression of fluorescent Venus protein-fused XBP1. The XBP1-ΔDBD, a deletion mutant of XBP1 lacking the DNA-binding domain, is also spliced by IRE1 in the same manner.

FIGURE 3. Messenger RNA of XBP1 is spliced by activated IRE1 in UPR. Under normal conditions, splicing of XBP1 mRNA by IRE1 does not occur, and immature XBP1 protein is produced. Under ER stress conditions, splicing by IRE1 occurs, followed by expression of fluorescent Venus protein-fused XBP1. The XBP1-ΔDBD, a deletion mutant of XBP1 lacking the DNA-binding domain, is also spliced by IRE1 in the same manner.
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Immunoblot analysis indicated that overexpression of wt-VAPB triggered expression of fluorescent XBP1-Venus protein (Fig. 4A, lane 4), whose expression was also confirmed by Venus fluorescence (Fig. 4B, wt). In contrast, expression of P56S-VAPB was unable to induce UPR when the same amount of plasmid was transfected (Fig. 4, A and B, P56S). Note again that the major fraction of P56S-VAPB was insoluble in the non-ER compartments (Fig. 4A, insoluble VAPB).

In a similar fashion, overexpression of wt-VAPA, but not that of P56S-VAPA, triggered UPR (data not shown). In contrast, overexpression of the other synaptic vesicular proteins, VAMP1 or VAMP2, was unable to induce UPR (Fig. 4, C and D), indirectly indicating that overexpressed wt-VAPB-mediated induction of UPR did not occur due to the simple overload of a synaptic vesicular protein but mimicked physiological UPR that may be mediated by wt-VAPB.

To examine the significance of the proline residue at the 56th position of VAPB in UPR induction, we performed a UPR induction assay using several VAPB mutants whose 56th amino acid was replaced. As shown in Fig. 4E, no tested P56X-VAPB derivatives (P56S, P56A, P56K, and P56D) nor P56del-VAPB (in which proline at position 56 is deleted) triggered UPR. In agreement, all of them showed increased Triton X-100 insolubility (Fig. 4E, bottom). These data support our hypothesis that the proline residue at the 56th position is essential for UPR induction by VAPB. In accordance, we recognized by immunofluorescence studies that P56A-VAPB or P56del-VAPB shows non-ER localization that is similar to that of P56S-VAPB (Fig. 4F). We also recognized that any other tested P56X-VAPB mutants showed subcellular localization almost similar to P56S- or P56A-VAPB.

Two amino acid substitutions of Scs2p and VAPA (K84D/L86D for...
P56S-VAPB Inhibits wt-VAPB-mediated UPR by Inducing the Insolubility of wt-VAPB

It thus appears that the P56S-VAPB mutant is a loss-of-function mutant in induction of UPR (Fig. 4). On the other hand, P56S-VAPB kept its ability to interact with wt-VAPB and also other synaptic vesicular proteins (Fig. 6). Considering that P56S-VAPB tends to be highly insoluble and that wt-VAPB and the other synaptic vesicular proteins are dimerized with P56S-VAPB, we hypothesized that wt-VAPB and the other synaptic vesicular proteins may be trapped in the insoluble fraction composed of P56S-VAPB and may lose their normal function when they are co-expressed.

To test this hypothesis, we examined whether P56S-VAPB altered the solubility of wt-VAPB or other synaptic vesicular proteins when they were co-expressed. As shown in Fig. 7A, co-expression of P56S-VAPB increased the amount of the insoluble His<sub>6</sub>-Xpress-tagged wt-VAPB and resulted in high molecular weight aggregates of wt-VAPB that were similar to misfolded and insoluble P56S-VAPB (Fig. 2B). In contrast, no increase in insolubility and misfolding of wt-VAPA, VAMP1, or VAMP2 was induced by co-expression of P56S-VAPB (Fig. 7, B–D), indicating that co-expression of P56S-VAPB specifically increases misfolding and insolubility of wt-VAPB. We have also demonstrated that any P56X-VAPB derivative (P56S, P56A, P56K, or P56D) or P56del-VAPB, but not K87D/M89D-VAPB, insolubilized co-expressed wt-VAPB, as shown in Fig. 7E.

To confirm this notion, we further performed sucrose density gradient centrifugation to investigate how P56S-VAPB captures co-expressed wt-VAPB. As shown in Fig. 7F, when cotransfected with the backbone vector, wt-VAPB co-

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Characterization of ALS-linked P56S-VAPB

Such an effect of P56S-VAPB on the co-expressed wt-VAPB thus appears to reduce the wt-VAPB function dominant negatively. To test this idea, NSC34 cells were cotransfected with the pEF1-vector or pEF1-P56S-VAPB in association with pCAX-F-XBP1-ΔDBD. Forty-eight h after transfection, they were incubated with 100 nm thapsigargin for six h. Subsequent UPR analysis indicated that UPR to thapsigargin-induced ER stress was attenuated, once NSC34 cells were transfected with P56S-VAPB (Fig. 8, A and B), supporting the idea that P56S-VAPB suppressed UPR, mediated by wt-VAPB, in a dominant negative manner.

The P56S Mutation Affects the Homodimerization of VAPB—As shown in Fig. 7, P56S-VAPB specifically affects the insolubility of co-expressed wt-VAPB but not that of wt-VAPA, VAMP1, or VAMP2. We therefore speculated that the protein-protein interaction mechanism underlying the VAPB homodimerization may be different from that underlying the heterodimerization between VAPB and any other synaptic vesicular protein. Several groups have demonstrated that a deletion of the C-terminal TMD from VAPB disables homodimerization or heterodimerization with other synaptic vesicle proteins (15, 21). We have observed that the deletion of C-terminal TMD disables VAPB from localizing in the membrane of ER or the Golgi apparatus. Therefore, it is possible that the deletion of C-terminal TMD attenuates the homodimerization or the heterodimerization by precluding proper localization of VAPB in the membrane of ER or the Golgi apparatus. As shown in Fig. 9A, however, we have also recognized that there is a weak interaction between wt-VAPB-ΔTMD and wt-VAPB, suggesting that VAPB may also form a homooligomer via sites other than the TMD. Furthermore, the P56S mutation of VAPB-ΔTMD dramatically enhanced the dimerization between P56S-VAPB-ΔTMD and P56S-VAPB (Fig. 9B), whereas it did not induce a similar effect for the dimerization between P56S-VAPB-ATMD and VAMP1 or VAMP2 (Fig. 9C). These data suggest that the P56S mutation may enable VAPB to form a stronger and more specific homodimer via the non-TMD site.

DISCUSSION

Elucidation of disease-causative abnormalities as well as the normal functions of ALS-linked genes has been steadily aiding understanding of the pathomechanisms underlying ALS. In this study, we have demonstrated that overexpression of wt-VAPB triggers UPR and that siRNA-mediated knockdown of endogenous VAPB expression attenuates Ire1/XBP1 signaling, triggered by DTT or thapsigargin. These findings strongly support the notion that VAPB is physiologically involved in UPR. The P56S mutation in the {VAPB/ALS8} gene causes the insolubility in the Triton X-100-containing lysis buffer and the localization shift of VAPB to non-ER compartments. As a result, it causes loss of function to induce UPR. Furthermore, P56S-VAPB enhances misfolding of co-expressed wt-VAPB and attenuates endogenous VAPB-mediated UPR. Taking together these findings and the clinical finding indicating that P56S-VAPB/ALS8

distributed with ER proteins, represented by calreticulin and calnexin. However, when cotransfected with P56S-VAPB, a considerable portion of wt-VAPB was distributed in the pellet fraction (Fig. 7G), supporting the notion that P56S-VAPB interferes with the wt-VAPB function by inducing the insolubility and misfolding of wt-VAPB as well as the localization shift of wt-VAPB to the non-ER and non-raft compartments. Immunofluorescence studies have confirmed that co-expression of P56S-VAPB induced localization shift of a considerable portion of wt-VAPB from ER to non-ER compartments (Fig. 7H).

4 K. Kanekura, I. Nishimoto, S. Aiso, and M. Matsuoka, unpublished data.
causes autosomal dominant ALS/SMA, we could hypothesize that the overall malfunction of VAPB-mediated UPR, caused by the P56S mutation in a single allele, may eventually lead to the abnormal accumulation of misfolded proteins in ER that contributes to the development of motoneuronal cell death related to ALS8. However, it is also possible that the loss of VAPB function in UPR by the P56S mutation may be unrelated to the onset of ALS/SMA. In the latter case, it should be hypothesized that the P56S mutation causes the gain of a new neurotoxic function. This issue should be addressed in future investigations.

In mammalian cells, there are three major UPR pathways: PERK, ATF6, and IRE1/XBP1 pathways (32). The IRE1/XBP1 (IRE1/HAC1 in yeast) pathway, the most conserved UPR pathway from yeast to mammals, increases protein chaperone expression and lipid synthesis (19). Based on the
Characterization of ALS-linked P56S-VAPB

observations that 1) IRE1 plays an essential role in both UPR induction and inositol metabolism (33); 2) disruption of either IRE1 or HAC1 causes inositol auxotrophy (34, 35); 3) overexpression of the yeast VAP homologue protein, Ssc2p, can rescue the inositol auxotrophy by IRE1/HAC1 mutants (36); and 4) mature XB1p increases lipid production (36), it has been assumed that there is a close relationship between UPR and lipid metabolism and that VAPB functionally associates with IRE1/XBP1 signaling. In support of this notion, it was reported that deprivation of inositol triggers UPR in yeast cells (38) and that SCS2 and HAC1 functionally interact with each other under UPR-inducing conditions (38). Considering that VAPA interacts with oxysterol-binding protein-related proteins (22, 39–41) and yeast Ssc2p interacts with yeast oxysterol-binding protein homologues, Osh1p and Osh2p (42), we speculate that oxysterol-binding protein-related proteins may be involved in VAPB-mediated UPR. The FFAT motif, whose consensus amino acid sequence is EFFDAXE, is conserved in a large protein family of oxysterol-binding protein-related proteins. As shown in Fig. 4E, the K87D/M89D mutation that has been demonstrated to prevent VAP and SCS2 from interacting with the FFAT motif-containing protein, resulting in loss of their functions (31), inhibited VAPB from triggering UPR, supporting our hypothesis. It was also reported that constitutive interaction between VAPA and oxysterol-binding protein, induced by a mutation in oxysterol-binding protein, causes the accumulation of unfolded proteins and ceramide in ER (22).

Amino acid substitution analysis demonstrated the importance of the proline residue at the position 56 for the function and the localization of VAPB. Based on the fact that the proline residue is an imino acid that restricts the flexibility of the peptide chain and plays a pivotal role in the correct conformation of the protein, an amino substitution of proline residue often causes a drastic conformational change, promoting functional abnormalities (43, 44). Therefore, it is assumed that the replacement or the deletion of the proline residue at position 56 in VAPB probably causes abnormal protein folding and insolubility that results in the localization shift to the non-ER fraction.

We also found a unique dominant negative effect of P56S-VAPB. It insolubilizes co-expressed wt-VAPB and weakens UPR, mediated by endogenous wt-VAPB (Figs. 7 and 8). By sucrose density gradient fractionation analysis and immunofluorescence analysis, we have confirmed that P56S-VAPB causes a shift of co-expressed wt-VAPB to the Triton X-100-insoluble non-ER fraction (Fig. 7G). Such insolubilization of co-expressed proteins specifically occurs when wt-VAPB, but not VAPA, VAMP1, or VAMP2, is co-expressed with P56S-VAPB (Fig. 7, A–D). Based on the finding that the P56S mutation enhanced the homodimerization of VAPB via the non-TMD site (Fig. 9B), we speculate that the dimerization between P56S-VAPB and wt-VAPB may be also specifically enhanced in a manner different from the homodimerization mediated through the C-terminal TMD. However, because it is extremely difficult to increase the expression level of soluble P56S-VAPB to the level of soluble wt-VAPB, this idea has not been experimentally proved.

The molecular mechanism underlying P56S-VAPB-induced motoneuronal death remains completely unknown. A straightforward hypothesis is that the malfunction of VAPB, induced by the P56S mutation, causes insufficient UPR induction, resulting in accumulation of misfolded proteins that may be toxic to motoneuronal cells in some situations. We need further in vitro and in vivo investigations to address the question of whether and how the insufficiency of VAPB functions is linked to motoneuronal degeneration.
