A mutation in VAPB that causes amyotrophic lateral sclerosis also causes a nuclear envelope defect

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Summary

A proline to serine mutation (P56S) in vesicle-associated membrane protein-associated protein B and C (VAPB) causes an autosomal dominant form of amyotrophic lateral sclerosis (ALS). We show that the mutation also causes a nuclear envelope defect. Transport of nucleoporins (Nups) and emerin (EMD) to the nuclear envelope is blocked, resulting in their sequestration in dilated cytoplasmic membranes. Simultaneous overexpression of the FFAT motif (two phenylalanine residues in an acidic track) antagonizes the effect of mutant VAPB and restores transport to the nuclear envelope. VAPB function is required for transport to the nuclear envelope, with knockdown of endogenous VAPB recapitulating this phenotype. Moreover, we identified the compartment into which the Nups and EMD were sequestered as the endoplasmic reticulum (ER)–Golgi intermediate compartment (ERGIC), with nuclear envelope membrane proteins transiting to the ERGIC before VAPB-dependent retrograde transport to the nuclear envelope.

Key words: Amyotrophic lateral sclerosis, VAPB, Nuclear envelope, gp210, Nup214, Emerin, ERGIC

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease caused by death of motor neurons. Familial ALS8 is caused by a P56S mutation in the vesicle-associated membrane protein-associated protein B and C (VAPB) gene (Nishimura et al., 2004; Chen et al., 2010). Overexpression of the VAPB P56S mutant protein leads to formation of large endoplasmic reticulum (ER)-derived membranes (Nishimura et al., 2004; Kanekura et al., 2006; Teuling et al., 2007; Prosser et al., 2008; Suzuki et al., 2009). VAPB is an integral membrane protein with an N-terminal major sperm protein (MSP) domain and a C-terminal transmembrane domain. The cytoplasmic MSP domain interacts with the FFAT motif (two phenylalanine residues in an acidic track) (Loewen et al., 2003) found in the oxysterol-binding protein (OSBP or ORP) family (Wyles et al., 2002), NIR phospholipid transfer proteins (Lev, 2004) and ceramide transport protein (Kawano et al., 2006). Coordinated membrane recruitment of these proteins by VAPs is thought to regulate lipid composition at membrane contact sites that in turn affects organelle morphology (Amarilio et al., 2005; Peretti et al., 2008). Replacement of P56 with a serine residue exposes a hydrophobic patch (Furuita et al., 2010; Kim et al., 2010) that renders the protein highly prone to aggregation (Kanekura et al., 2006). We have previously shown that co-overexpression of an FFAT-containing fragment, but not when the phenylalanine residues were replaced with alanine residues (named AAAT), rescued the abnormal ER morphology induced by mutant VAPB (Prosser et al., 2008). Here, we report that VAPB P56S mutation also causes a nuclear envelope defect characterized by separation of the outer and inner nuclear membrane (ONM and INM, respectively). This defect is caused by disruption of transport of nuclear envelope proteins because loss of VAPB led to their accumulation in dilated cytoplasmic membranes viewed under light microscopy in cells expressing mutant VAPB alone or together with AAAT were large cytoplasmic vacuole-like structures and a dilated or ‘herniated’ nuclear envelope (Fig. 1A–C). The vacuole-like structures probably correspond to the dilated membranes viewed under light microscopy in cells expressing mutant VAPB (Teuling et al., 2007; Fasana et al., 2010), and these were also evident in some cells. However, the most prominent features in cells coexpressing mutant VAPB alone or together with AAAT were large cytoplasmic vacuole-like structures and a dilated or ‘herniated’ nuclear envelope (Fig. 1A–C). The vacuole-like structures probably correspond to the dilated membranes viewed under light microscopy in cells expressing mutant VAPB (Teuling et al., 2007; Fasana et al., 2010). Interestingly, ∼75% of these cells also showed a prominent nuclear envelope defect characterized by separation of the INM and ONM at discrete regions (Fig. 1A, arrows). The gap between the two membranes can be as much as 500 nm apart, with a mean of 160±32 nm, which is over twofold greater than the 70±5 nm gap in control cells (Fig. 1D). Connecting ER tubules, when detected, also appeared dilated (Fig. 1C, #). These ER and nuclear envelope defects were not observed in cells co-overexpressing mutant VAPB and the FFAT fragment, consistent with the ability of this motif to rescue the adverse effects of the mutant protein. Thus, overexpression of mutant VAPB not only causes formation of
aberrant cytoplasmic membranes but also results in separation of the two nuclear membranes.

Retention of Nups and EMD in mutant VAPB-induced dilated membranes

We next examined mislocalization of nucleoporins (Nups) as a possible cause of the defects because nuclear pores spanning the two nuclear membranes help maintain their close apposition. We examined two Nups with vastly different membrane topology and residence times at the nuclear envelope; gp210 (also known as Pom210) is a highly dynamic integral membrane protein of the nuclear pore complex (NPC), whereas Nup214, which forms the structural scaffold, has a nuclear envelope residence time an order of magnitude higher than gp210 (Daigle et al., 2001; Rabut et al., 2004). In cells transfected with empty vector and wild-type VAPB (VAPB-WT), gp210–GFP formed a ring-like pattern encompassing the rim of the nuclear envelope, as well as being localized to scattered cytoplasmic puncta (Fig. 2A), consistent with previous studies. Cytoplasmic gp210–GFP colocalized extensively with VAPB in tubular membranes. Its nuclear envelope localization time an order of magnitude higher than gp210 (Daigle et al., 2001; Rabut et al., 2004). In cells transfected with empty vector and wild-type VAPB (VAPB-WT), gp210–GFP formed a ring-like pattern encompassing the rim of the nuclear envelope, as well as being localized to scattered cytoplasmic puncta (Fig. 2A), consistent with previous studies. Cytoplasmic gp210–GFP colocalized extensively with VAPB in tubular membranes. Its nuclear envelope localization was unaffected by up to a threefold overexpression of the transgene, relative to endogenous VAPB. By contrast, gp210–GFP was excluded from the nuclear envelope and retained in mutant-VAPB-containing dilated cytoplasmic membranes (Fig. 2A). Similarly, Nup214–GFP, which normally localizes to the nuclear envelope, was sequestered in mutant-VAPB-containing cytoplasmic membranes, although not all mutant-VAPB-containing membranes were Nup214–GFP-positive (Fig. 2B). This effect was not cell line specific, as HeLa cells had a similar Nup214 relocation defect (supplementary material Fig. S1). The normal nuclear envelope localization of both gp210–GFP and Nup214–GFP were restored upon co-overexpression of the FFAT fragment (Fig. 2A,B), consistent with the ability of this motif to rescue the effects of mutant VAPB. Thus, cytoplasmic retention of these Nups along with aggregated mutant VAPB inhibits their transport to the nuclear envelope.

To determine whether trafficking of other nuclear envelope proteins is also affected, we examined the distribution of emerin (EMD), an integral membrane protein that shuttles between the ER and INM (Zuleger et al., 2011). Endogenous EMD formed a ring-like pattern along the rim of the nuclear envelope in both control and VAPB-WT-transfected cells (Fig. 2C). It partially colocalized with VAPB-WT in cytoplasmic puncta throughout the cell as well as in discreet regions adjacent to or at the nuclear envelope. By contrast, EMD was retained in dilated cytoplasmic membranes and excluded from the nuclear envelope in cells overexpressing mutant VAPB (Fig. 2C). These EMD-containing membranes were often positioned adjacent to but not part of the nuclear envelope. Co-overexpression of the FFAT fragment with VAPB-WT had no effect on EMD distribution, but restored EMD localization to the nuclear envelope in mutant VAPB cells (Fig. 2C). Because EMD is retained in the INM in part by binding to A-type lamins in the nuclear lamina (Vaughan et al., 2001; Ostlund et al., 2006), we examined the distribution of lamin A/C and found no change in its distribution pattern or evidence of nuclear deformation (data not shown). Thus, loss of EMD from the INM is probably due to disruption of transport to the INM rather than loss of retention at the INM.

Cytoplasmic retention is due to loss of VAPB function

Mutant VAPB is aggregation-prone, and recruitment of endogenous VAPB to insoluble aggregates is thought to result in a dominant-negative effect (Teuling et al., 2007; Suzuki et al., 2009; Kim et al., 2010). To determine whether the defect is due to loss of VAPB function and to exclude non-specific sequestration of nuclear envelope proteins with aggregated
mutant VAPB, we examined their distribution upon siRNA knockdown of endogenous VAPB (siVAPB). Co-transfection with the empty pLKO.1 vector had no effect on the distribution pattern of Nup214–GFP, whereas siVAPB resulted in relocation of Nup214–GFP to cytoplasmic foci and loss of nuclear envelope localization (Fig. 3A). The cytoplasmic foci probably represent sites of NPC assembly because Nup214 is a soluble protein and recruited to the membrane to form the core scaffold during NPC assembly (Bodoor et al., 1999). The accumulation of Nup214–GFP suggests that VAPB is required for transport of the pre-assembled NPC to the nuclear envelope. It is consistent with the view that mutant VAPB acts in a dominant negative manner.

Transport of EMD was similarly inhibited by siVAPB. Endogenous EMD was relocated from the nuclear envelope to large cytoplasmic puncta throughout the cell upon siVAPB (Fig. 3B). Thus, VAPB function is essential for transport of Nup214, EMD and possibly other membrane proteins to the nuclear envelope. Loss of nuclear envelope localization is not simply due to inadvertent sequestration of Nups with mutant VAPB aggregates but a consequence of loss of VAPB function.

Fig. 2. Nuclear envelope proteins are retained in mutant VAPB-containing membranes. CHO cells were co-transfected with FLAG-tagged VAPB (WT or P56S) and gp210–GFP (A) or Nup214–GFP (B). FFAT co-transfected with mutant VAPB is shown in the bottom panels. (C) Cells were transfected with FLAG-VAPB-WT or P56S and stained with anti-FLAG (red) and anti-EMD (green) antibodies. FFAT was co-transfected with mutant VAPB in the bottom panel. Scale bars: 10 μm. All images are representative of at least 90% of transfected cells from three replicates.
VAPB affects ERGIC trafficking

To identify the VAPB-containing intracellular compartment, we examined its co-localization with known organelle markers. Only cells with a low level of expression of transfected VAPB-WT were examined to avoid potential overexpression artifacts. We found VAPB-WT colocalized extensively with GFP-ERGIC-53-containing cytoplasmic puncta (Fig. 4A). The perinuclear Golgi ribbon was devoid of VAPB, suggesting that VAPB primarily resides in the ERGIC. When subjected to intensity correlation analysis, excluding the Golgi ribbons, VAPB and ERGIC-53 showed a mean Pearson's correlation of 0.73 ± 0.01 and 0.79 ± 0.02 (from three replicates of 10–17 cells), respectively. These high coefficients suggest colocalization with recycling ERGIC-53. To determine whether VAPB affects trafficking through the ERGIC, we examined the distribution of endogenous ERGIC-53 upon siVAPB transfection. ERGIC-53 relocated from the Golgi to expanded membranes in 45.6% ± 0.9% (n = 3) of siVAPB cells (Fig. 4B) compared with 4.9% ± 1.9% in pLKO.1 controls, indicating that relocation was not an artifact of ectopic ERGIC-53 expression. Quantification of individual peripheral cytoplasmic puncta indicated a shift towards larger ERGIC-53 puncta in the knockdown cells (Fig. 4C). This indicates that VAPB is essential to maintain ERGIC morphology and retrograde trafficking of ERGIC-53. To verify that Nups are retained in ERGIC in a VAPB-dependent manner, we stained the knockdown cells with mAb414, a monoclonal antibody that recognizes several FG repeat Nups (Davis and Blobel, 1986). These Nups were normally localized to the rim of the nuclear envelope and in small cytoplasmic puncta in control cells, but were retained in expanded GFP-ERGIC-53-containing membranes upon siVAPB transfection (Fig. 4C). Distribution of EMD was similarly affected, relocating to the expanded ERGIC along with GFP-ERGIC-53 in siVAPB cells (Fig. 4D). Quantification of cytoplasmic puncta staining with mAb414 and containing EMD, excluding the nuclear envelope, also indicated a shift towards larger puncta in the knockdown cells (Fig. 4C). Taken together, these results indicate that nuclear envelope and transmembrane Nups transit through ERGIC, and VAPB is required for final transport to the nuclear envelope.

Our study shows that overexpression of mutant VAPB not only causes ER abnormalities but also separation of the ONM from the INM at discreet regions of the nuclear envelope. Interestingly, this abnormality is similar to that caused by disruption of gp210 (Drummond and Wilson, 2002), which leads to formation of nuclear pore intermediates that fail to dilate into functional pores. This suggests that positioning of fully assembled pores at the junction of the two nuclear membranes structurally contributes to their close apposition. Cytoplasmic retention of certain Nups and membrane proteins upon siRNA knockdown clearly indicates that VAPB is of essential role in their transport to the nuclear envelope. This disruption in transport will probably affect the overall composition of the NPC and compromise the structural integrity as well as functional properties of the pores. Interestingly, bulk anterograde ER-to-Golgi transport of vesicular stomatitis virus G protein (VSVG) remains largely unaffected upon siVAPB (supplementary material Fig. S2), suggesting that VAPB is involved in regulating retrograde transport through ERGIC.

Co-overexpression of the FFAT fragment clearly counteracts the adverse effects of mutant VAPB. We propose that interaction with the FFAT fragment might induce changes that reduce aggregate formation, allowing partial restoration of wild-type functions and averting aggregate toxicity. Binding to the FFAT fragment might impart conformational changes that reduce aggregate formation, allowing partial restoration of wild-type functions and averting aggregate toxicity. Cytoplasmic retention of Nups and EMD in mutant VAPB-containing membranes is not due to inadvertent sequestration with insoluble aggregates because siRNA knockdown of endogenous VAPB also results in their cytoplasmic retention. Given that wild-type VAPB colocalizes with recycling ERGIC-53, and siVAPB knockdown results in expansion and retention of Nups and EMD with ERGIC-53, it suggests that these nuclear envelope membrane proteins do not reach the nuclear envelope by lateral diffusion through the interconnecting ER and ONM network, but transit through ERGIC. Progression from these ERGIC foci is clearly dependent on VAPB with its loss of function either through siRNA knockdown or dominant negative
effect of mutant VAPB overexpression inhibiting their exit and consequently expansion of ERGIC. While the mechanism by which VAPB facilitates this retrograde transport step remains to be determined, progressive deterioration of the nuclear envelope is a predictable consequence of disrupting this transport step and could contribute to age-dependent onset of the disease.

Materials and Methods

Expression plasmids, cell culture and transfection
FLAG-tagged VAPB and Myc-tagged FFAT constructs were as described previously (Prosser et al., 2008). Gp210–GFP and Nup214–GFP were from EUROSCARF. Chinese Hamster Ovary (CHO-K1) and HeLa cells were maintained at 37°C in MEMα and DMEM (Invitrogen, Carlsbad, CA, USA), respectively, and supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). Cells were transfected with LipofectAMINE (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

Immunofluorescence and electron microscopy
Transfected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature. After neutralization with PBS-glycine (PBS with 100 mM glycine), cells were permeabilized in blocking buffer (PBS, 1% bovine serum albumin, 2% normal goat serum, 0.4% saponin and 0.1% Triton X-100). Primary and secondary antibodies were diluted in blocking buffer and incubated for at least 1 hour. Cells were mounted onto coverslips with SlowFade Gold with or without DAPI (Invitrogen, Carlsbad, CA, USA). Primary antibodies used included anti-FLAG (Applied Biological Materials, Richmond, BC, Canada), mAb414 (Covance, Princeton, NJ, USA), anti-ERGIC-53 (Sigma-Aldrich, St Louis, MO, USA) and anti-EMD (MANEM1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Secondary antibodies were conjugated with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA, USA).

Images were captured on a LSM 510 confocal microscope with a 1.4 numerical aperture 63× oil-immersion objective and processed with Image J (NIH, Bethesda, MD, USA). Sizes of ERGIC-53, mAb414 and EMD cytoplasmic particles, excluding the perinuclear Golgi ribbons and nuclear envelope, were measured with the particle analysis plug-in. Over 10 cells were chosen from each group and from...
three replicates. The maximal Feret’s diameter of individual peripheral puncta (>200 per cell) were grouped into 100 nm bins. An unpaired two-tailed Student’s t-test was used to determine statistical significance.

For electron microscopy, cells were fixed with 1.6% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 2–6 hours. After contrasting with 1% osmium tetroxide and dehydration in increasing concentrations of ethanol, cells were embedded in Spurr’s low viscosity epoxy (Polysciences, Warrington, PA, USA). Resulting ultra-thin sections were stained with 5% uranyl acetate for 15 minutes and Reynold’s Lead Citrate Solution for 5 minutes. Digital images were taken with a JEOL 1230 transmission electron microscope at 60 kV adapted with a 2000×2000 pixel bottom mount Hamamatsu CCD digital camera.

Lentiviral pLKO.1-based plasmids were from Open Biosystems (Hunstville, AL, USA). TRCN0000153862 and TRCN0000152888 matched both human and mouse VAPB sequences. Empty pLKO.1 vector or eGFP shRNA were used as controls. In some cases, co-transfection with monomeric RFP (mRFP) was used to identify the transfected cells. Cells were processed 48 hours post transfection.

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Supplementary material available online at

References


Fig. S1. Cellular distribution of Nup214 in HeLa cells. HeLa cells were co-transfected with FLAG-VAPB (WT or P56S), and Nup214-GFP. Cells were fixed after 48h and stained with anti-FLAG antibodies. Scale bar, 10 µm.

Fig. S2. Transport of anterograde VSVG\textsuperscript{ts042}-GFP in siVAPB knockdown cells. (A) HeLa cells were co-transfected with pLKO.1 empty vector or siVAPB and VSVG\textsuperscript{ts042}-GFP. VSVG\textsuperscript{ts042}-GFP was trapped in the ER at 42°C for 5 h, and shifted to 32°C for the time indicated before fixation. (B) Fraction of cells with Golgi-localized VSVGts042-GFP after release from 42°C. Error bars represent s.e.m. (n=3).