Inclusion body myopathy-associated mutations in p97/VCP impair endoplasmic reticulum-associated degradation

Conrad C. Weihl1,2,*, Seema Dalal2, Alan Pestronk1 and Phyllis I. Hanson2

1Department of Neurology and 2Department of Cell Biology and Physiology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA

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Mutations in the AAA+ protein (ATPase associated with a variety of cellular activities) p97/VCP (valosin-containing protein) cause a dominantly inherited syndrome of inclusion body myopathy with Paget’s disease of the bone and fronto-temporal dementia (IBMPFD). p97/VCP is a ubiquitously expressed protein that participates in a number of cellular processes including endoplasmic reticulum-associated degradation (ERAD). p97/VCP aids in the extraction of ubiquitinated proteins from the endoplasmic reticulum (ER) and facilitates their delivery to the proteasome. This study focuses on the effects of disease-associated p97/VCP mutations on this pathway. We show that p97/VCP containing the most prevalent IBMPFD-associated mutation, R155H, has normal ATPase activity and hexameric structure. However, when expressed in cultured cells, both this and a second IBMPFD-associated p97/VCP mutant increase the overall level of ubiquitin-conjugated proteins and specifically impair degradation of mutant ΔF508-CFTR handled by the ERAD pathway. These effects are similar to those previously described for an ATPase deficient p97/VCP mutant and suggest that IBMPFD mutations impair p97/VCP cellular function. In a subset of cells, IBMPFD mutations also promote formation of aggregates that contain p97/VCP, ubiquitin conjugates and ER-resident proteins. Undegraded mutant ΔF508-CFTR also accumulates in these aggregates. We conclude that IBMPFD mutations in p97/VCP disrupt ERAD and that this may contribute to the pathogenesis of IBMPFD.

INTRODUCTION

Inclusion body myopathies (IBMs) are hereditary and sporadic disorders that produce weakness and are characterized by muscle pathology that includes rimmed vacuoles and cytoplasmic aggregates (1). Cytoplasmic aggregates in muscle in these disorders contain ubiquitin, β-amyloid, apolipoprotein E and phosphorylated tau, the same proteins that accumulate in Alzheimer’s disease brains (2). One form of hereditary IBM, an autosomal dominantly inherited syndrome that also presents with Paget’s disease of the bone and fronto-temporal dementia (IBMPFD), is caused by missense mutations in the gene encoding the protein p97/VCP (valosin-containing protein) (3). Mutations in p97/VCP cause cytoplasmic and nuclear aggregates in IBMPFD muscle and brain tissues, respectively (3,4). The identity of other protein components of the aggregates has not been defined.

p97/VCP belongs to the AAA+ (ATPases associated with a variety of cellular activities) family of chaperone-like proteins. AAA+ proteins are involved in many cellular processes including membrane trafficking, organelle biogenesis, DNA replication, protein degradation and protein folding (5–7). p97/VCP is involved directly or indirectly in several of these processes (8,9). One role for p97/VCP is in the ubiquitin–proteasome system, where p97/VCP has been shown to associate with polyubiquitinated proteins, the 26S proteasome, ubiquitin ligases gp78 and Ufd2, deubiquitinating enzymes VCIP135 and ataxin-3 and several other ubiquitin-like and ubiquitin-interacting proteins (9–15). p97/VCP may help coordinate the activity of these different factors and participate in the delivery of ubiquitin-conjugated proteins to the proteasome for degradation.

The most clearly defined role of p97/VCP is in removing improperly folded proteins from the ER for degradation by
the proteasome, a process known as endoplasmic reticulum-associated degradation (ERAD) (16,17). In particular, p97/VCP associates with derlin-1 (18,19). Derlin-1 has been proposed to be the channel through which misfolded proteins are extruded from the ER lumen and membrane. p97/VCP may couple derlin-1 to proteasome pathway components allowing ERAD to occur.

p97/VCP-related pathology has been identified in several disease syndromes in which it is physically associated with aberrantly folded proteins. In particular, p97/VCP is found in Lewy bodies in diffuse Lewy body and Parkinson’s disease, huntingtin protein-positive intranuclear inclusions in Huntington’s disease, ubiquitinated inclusions in motor neuron disease, Mallory bodies from cirrhotic livers and eosinophilic aggregates in sporadic inclusion body myositis (3,20–22). p97/VCP also binds to aggregated polyglutamine-containing proteins (21). The role of p97/VCP in the pathogenesis of these disorders remains unclear, but association with misfolded or aggregated proteins is a common theme among them.

p97/VCP contains three domains, an N-domain involved in ubiquitin and cofactor binding and two tandem AAA+ domains, D1 and D2, which bind and hydrolyze ATP. p97/VCP subunits assemble into functional hexamers, with a central cylinder formed by the AAA+ domains and surrounded by N-domains (23). IBMPFD disease causing mutations are located in three different regions of the protein. Most mutations, including the most prevalent R155H mutation as well as changes of R95G, R155P and R155C, are located within the N-domain, one (R191Q) is within the linker connecting N- and D1-domains and one (A232E) is within the D1-domain (3). The effect of mutations in these particular residues on the structure and function of p97/VCP remains to be elucidated.

In this study, we explored the effects of IBMPFD mutations in p97/VCP on its intrinsic enzymatic activity, as well as its subcellular distribution and function in ERAD when expressed in cultured C2C12 myoblasts and U2OS osteosarcoma cells. We conclude that IBMPFD-associated mutations in p97/VCP disrupt the turnover of ubiquitin-conjugated proteins particularly at the ER membrane and in some cases accumulate together with these proteins in aggregates that are adjacent to or on the ER. This effect is not due to a loss of basal ATPase activity. Instead, the mutations may alter protein–protein interactions with specific components of the ubiquitin–proteasome and ERAD pathways to generate dysfunctional and in some cases aggregated p97/VCP.

RESULTS
IBMPFD mutant p97/VCP forms intact hexamers and has normal ATPase activity

We first examined the structure of p97/VCP oligomers containing the most common IBMPFD mutation, a missense mutation that replaces arginine 155 in the enzyme’s N-domain with a histidine (R155H) (3). Purified, bacterially expressed p97/VCP-R155H migrated as a single band of 97 kDa on SDS–PAGE. The mutant p97/VCP-R155H eluted from a size exclusion column in the same position as hexameric p97/VCP-WT, suggesting that it was properly assembled into a hexamer and not otherwise aggregated (not shown). Quick-freeze/deep-etch electron microscopy revealed intact hexamers of p97/VCP-WT (A) and p97/VCP-R155H (B). Arrows denote cylindrical protein structure typical of normal hexameric p97/VCP (40). (C) ATPase activity of purified recombinant p97/VCP-WT (diamonds), p97/VCP R155H (squares) or double mutant E305Q/E578Q (triangles) (which lacks ATPase activity) from four independent experiments.

Figure 1. IBMPFD mutant p97/VCP forms intact hexamers and has normal ATPase activity. Freeze-fracture/deep-etch electron microscopy of recombinant purified p97/VCP-WT (A) or p97/VCP-R155H (B). Arrows denote cylindrical protein structure typical of normal hexameric p97/VCP (40). (C) ATPase activity of purified recombinant p97/VCP-WT (diamonds), p97/VCP R155H (squares) or double mutant E305Q/E578Q (triangles) (which lacks ATPase activity) from four independent experiments.
the ATPase activity measured was specific to purified p97/VCP, we prepared p97/VCP-E305Q/E578Q (with mutations in both the D1- and D2-domains). Consistent with previous reports, this mutant had no activity (25).

**IBMPFD mutant p97/VCP impairs ERAD in cultured cells**

To evaluate the overall functioning of the ubiquitin–proteasome system in cells expressing IBMPFD mutant proteins, we examined the level and distribution of ubiquitinated proteins in cells transfected with different p97/VCP variants, using an antibody that recognizes only ubiquitin-conjugated proteins and not free ubiquitin (FK2) (26). Untransfected C2C12 cells had a low level of detectable ubiquitin conjugates (Supplementary Material, Fig. S1). When C2C12 cells were treated with the proteasome inhibitor, MG132, there was a notable increase in both diffuse and punctate FK2 immunostaining, indicating an increase in diffuse and aggregated ubiquitin-conjugated proteins (Supplementary Material, Fig. S1). Similar to untransfected

**Figure 2.** IBMPFD mutant p97/VCP increases ubiquitin-conjugated proteins in C2C12 cells. C2C12 cells were transiently transfected with vectors containing p97/VCP-WT-GFP, p97/VCP-R155H-GFP, p97/VCP-R95G-GFP or a dominant negative ATPase inactive p97/VCP-E578Q-GFP and immunostained with FK2 antibody to evaluate the level of ubiquitin-conjugated proteins. Confocal images of p97/VCP-GFP transfected cells (green, intrinsic p97/VCP-GFP fluorescence) and FK2-ubiquitinated aggregates (red) are shown. (A) p97/VCP-WT-GFP; (B–E) p97/VCP-R155H-GFP; (F and G) p97/VCP-R95G-GFP; (H) p97/VCP-E578Q-GFP. Closed arrows denote representative ubiquitin-conjugated aggregates. Open arrows denote co-localized p97/VCP aggregates and ubiquitinated aggregates. All images were taken with the laser power and gain held constant to allow direct comparison between cells.
C2C12 cells, p97/VCP-WT-GFP transfected C2C12 cells had low levels of ubiquitinated proteins (Fig. 2A). In contrast, transfection of C2C12 cells with disease-associated p97/VCP mutants (Fig. 2B–G) led to a prominent increase in both diffuse and aggregated ubiquitin conjugates in all cells. A similar increase in ubiquitin conjugates was seen when C2C12 cells were transfected with the dominant negative ATPase inactive mutant p97/VCP-E578Q-GFP (Fig. 2H) as previously described, using p97/VCP-E578Q-myc (25). These results indicate that each of the mutant proteins compromise p97/VCP function in the cellular ubiquitin pathway. As discussed subsequently, IBMPFD mutant p97/VCP-GFP-containing aggregates were present in a subset of the transfected cells and may indicate that IBMPFD mutations induce a subtle structural perturbation in p97/VCP. Some ubiquitinated aggregates contained p97/VCP-GFP (Fig. 2C–E and G, open arrows), whereas others did not (Fig. 2B–D and F–H, closed arrows).

As p97/VCP has been implicated in ERAD (27), we looked directly at the effect of IBMPFD mutants on the degradation of a well-characterized ERAD substrate, the ΔF508 mutant cystic fibrosis transmembrane regulator (ΔF508-CFTR) (28,29). Immunoblotting lysates prepared 36 h after co-transfecting p97/VCP-WT-GFP and ΔF508-CFTR into U2OS cells, using a CFTR-specific antibody demonstrated that this mutant CFTR is present at low levels in p97/VCP-WT-GFP expressing cells (Fig. 3A). However, when IBMPFD mutant p97/VCP-R155H-GFP or p97/VCP-R95G-GFP was co-transfected

Figure 2. Continued.
with ΔF508-CFTR, there was an increase in the level of ΔF508-CFTR suggesting that ERAD is impaired in these cells (Fig. 3A). A histogram representing densitometric analysis from three independent co-transfection experiments confirms the trend of an increase in undegraded ΔF508-CFTR in IBMPFD mutant p97/VCP-R155H or p97/VCP-R95G expressing cells compared with the amount of undegraded ΔF508-CFTR in p97/VCP-WT expressing cells (Fig. 3B). Immunofluorescence of similarly co-transfected cells demonstrated that ΔF508-CFTR co-localized with IBMPFD mutant p97/VCP-R155H and p97/VCP-R95G in aggregates (Fig. 3C and D, respectively). In co-transfected cells that do not contain p97/VCP aggregates, ΔF508-CFTR immunofluorescence was reticular consistent with its normal ER localization (Fig. 3C and D). These data support the hypothesis that IBMPFD mutant p97/VCPs impair the degradation of ERAD substrates although an alternative explanation would be that IBMPFD mutant p97/VCP increases ΔF508-CFTR expression.

**IBMPFD mutant p97/VCP aggregates in cultured cells**

The IBMPFD mutant p97/VCP aggregates seen in transfected C2C12 cells (Fig. 2) may be similar to p97/VCP immunoreactive aggregates in IBMPFD patient muscle tissue (3). In order to confirm that the IBMPFD p97/VCP aggregates described earlier were not an artifact of fixation, we looked at images of live cells expressing IBMPFD mutant p97/VCPs.
As a control, images of live p97/VCP-WT-GFP expressing C2C12 cells show that the wild-type protein is localized evenly throughout the cell, both in the nucleus and the cytoplasm (Fig. 4A). This distribution of p97/VCP-WT-GFP is similar to that of myc-tagged p97/VCP (25) and endogenous p97/VCP in C2C12 cells (Supplementary Material, Fig. S3).

In contrast, IBMPFD mutant p97/VCP-R155H-GFP and p97/VCP-R95G-GFP often formed large cytoplasmic aggregates that were concentrated around the nucleus (Fig. 4A). We counted ~300 transfected cells from three independent experiments and determined that 30% of cells expressing p97/VCP-R155H-GFP and 31% of cells expressing p97/VCP-R95G-GFP contained aggregates. Only 7% of p97/VCP-WT-GFP expressing cells had comparable aggregates (Fig. 4B). Similar p97/VCP aggregates were also seen when IBMPFD mutant p97/VCP was expressed in the human U2OS osteosarcoma cell line we used previously to study p97/VCP function (Fig. 4A) (25).

To see if IBMPFD mutant p97/VCP aggregates sequester p97/VCP-WT protein and might therefore potentially deplete the cell of its normal enzyme, we co-transfected U2OS cells with GFP-tagged IBMPFD mutants and myc-tagged p97/VCP-WT. Indirect immunofluorescence demonstrated that IBMPFD mutant p97/VCP proteins in aggregates, p97/VCP-R95G-GFP and p97/VCP-R155H-GFP, contained p97/VCP-WT-myc. (Fig. 4C and Supplementary Material, Fig. S3). This may be because mutant and wild-type p97/VCP aggregates are enriched in the insoluble fraction.

Figure 4. IBMPFD mutant p97/VCP aggregates in cultured cells. (A) Confocal images of p97/VCP-WT-GFP (WT), p97/VCP-R155H-GFP (R155H) or p97/VCP-R95G-GFP (R95G) transfected C2C12 (upper panels) or U2OS cells (lower panels). (B) Quantitation of transfected C2C12 cells containing p97/VCP aggregates. Approximately 300 transfected cells from three independent experiments were counted. Asterisks indicate differences with statistical significance of P < 0.001. (C) Co-assembly of mutant and wild-type p97/VCP in aggregates. U2OS cells were transiently transfected with p97/VCP-WT-myc and p97/VCP-R95G-GFP (R95G). Cells were immunostained with anti-myc antibody. Green shows green fluorescent protein (GFP)-tagged p97/VCP-R95G. Red shows p97/VCP-WT-myc immunoreactivity. Merge panel demonstrates co-localization of p97/VCP-WT and IBMPFD mutant p97/VCP proteins in aggregates. (D) Immunoblot of p97/VCP-WT-GFP, p97/VCP-R155H-GFP and p97/VCP-R95G-GFP transfected C2C12 cell lysates treated as follows. Unfractionated lysates, insoluble/pellet (P) and soluble/supernatant (S) fractions from mechanically lysed protein fractions were separated by SDS–PAGE and immunoblotted for GFP. Actin immunoblot from the same lysates is shown as a loading control. Immunoblot is representative of two independent transfection experiments. p97/VCP-WT-GFP is present in the soluble fraction, whereas p97/VCP-R155H-GFP and p97/VCP-R95G-GFP are enriched in the insoluble fraction.
proteins can co-assemble to form stable hexamers (25). Singly transfected cells with p97/VCP-WT-myc do not show evidence of p97/VCP aggregation (Supplementary Material, Fig. S3) (25).

To determine whether the visually defined aggregates of p97/VCP-GFP described earlier contain physically aggregated protein as seen in other aggregate diseases (30,31), we solubilized p97/VCP-WT-GFP, p97/VCP-R155H-GFP or p97/VCP-R95G-GFP transfected C2C12 cells with 0.5% Triton X-100 and separated soluble from insoluble proteins by centrifugation. Protein fractions were separated by SDS–PAGE and immunoblotted for p97/VCP-GFP, using an antibody against GFP. p97/VCP-WT-GFP was largely soluble while IBMPFD mutant p97/VCP-R155H-GFP and p97/VCP-R95G-GFP were present in both soluble and insoluble fractions (Fig. 4D). This difference is not due to differing protein expression levels, as total cell lysates show approximately the same amount of p97/VCP protein in each sample.

In order to further define the origin of the IBMPFD p97/VCP aggregates, we co-immunostained transfected cells with cellular organelle markers. The aggregates formed by IBMPFD-mutant p97/VCP did not co-localize with the Golgi apparatus protein giantin (Fig. 5A) and did not have the properties of aggresomes seen in other diseases of aggregated proteins (32). Aggresomes are large, single structures concentrated at the microtubule organizing center and rimmed by vimentin (32). p97/VCP aggregates are multiple, located around the nucleus and throughout the cytoplasm (Fig. 4A). Because p97/VCP is involved in ERAD (25), we looked at the distribution of two ER proteins, protein disulfide isomerase (PDI) and calnexin, in transfected cells. Both PDI and calnexin co-localize with p97/VCP in most of the aggregates although some p97/VCP aggregates are devoid of PDI or calnexin (Fig. 5B). p97/VCP-WT-GFP transfected cells do not typically have aggregates and have a normal ER-immunostaining pattern (Fig. 5B).

In order to evaluate the ultrastructure of IBMPFD mutant p97/VCP expressing cells, we prepared samples for examination by thin-section electron microscopy (Fig. 6). p97/VCP-R95G-GFP and p97/VCP-R155H-GFP transfected U2OS cells had significantly distorted ER, readily identified as dilated ribosome-studded membrane containing proteinaceous debris (Fig. 6B). In addition, electron-dense aggregates were present in the cytoplasm as well as adjacent to the ER membrane (Fig. 6B–D). Untransfected U2OS cells had normal appearing ER and few aggregates (Fig. 6A). These results suggest that IBMPFD mutant p97/VCPs alter ER structure and increase intracellular protein aggregate formation.

**DISCUSSION**

IBMPFD is an autosomal dominant syndrome with a myopathy that includes muscle fiber inclusions and vacuoles. It is caused by mutations in p97/VCP, a protein involved in diverse cellular processes including delivery of certain
proteins to the proteasome for degradation (3). p97/VCP and ubiquitin are present as aggregates in muscle from both IBMPFD and sporadic inclusion body myositis patients (3) (C.C.W. unpublished data). Disruption of the ubiquitin–proteasome pathway by dysfunctional p97/VCP may result in the accumulation and aggregation of undegraded misfolded proteins that is seen in muscle fibers in IBM (1).

This study explores the effects of two IBMPFD mutations on p97/VCP function in order to determine how the mutations might contribute to these problems. We demonstrate that the most common IBMPFD mutant p97/VCP (R155H) forms hexamers with normal basal ATPase activity in vitro. However, expression of both this and a second (R95G) IBMPFD mutant p97/VCP in cultured cells leads to a marked general increase in ubiquitin-conjugated proteins and impaired degradation of ERAD substrates. In addition, these mutations increase the propensity of p97/VCP to aggregate, and many of these aggregates are associated with ER membranes. We suggest that impairment in ERAD and/or aggregation of mutant IBMPFD proteins could contribute to the pathogenesis of IBMPFD.

Several lines of evidence demonstrate that IBMPFD mutations in p97/VCP affect the ubiquitin–proteasome pathway and more specifically ERAD. (i) IBMPFD mutant p97/VCPs increase the level of ubiquitin-conjugated aggregates in the cytosol and at the ER membrane. (ii) IBMPFD mutant p97/VCP expression generates distorted and swollen ER similar to that generated in response to ER stress (33,34). (iii) IBMPFD mutant p97/VCP expression slows the degradation of ΔF508-CFTR, a known ERAD substrate, and undegraded mutant CFTR co-localizes with IBMPFD mutant p97/VCP aggregates.

Figure 5. Continued.
It is unclear precisely how IBMPFD mutations in p97/VCP affect its function in ERAD. Likely mechanisms include one or more of the following. First, mutant p97/VCP may not be able to associate with co-factors essential for delivering ubiquitinated proteins from the ER to the proteasome. Secondly, mutations in p97/VCP may impair its ability to transmit conformational changes from the enzyme’s central AAA+ domains during ATP hydrolysis to the attached N-domains resulting in an inactive hexamer. Finally, mutations in p97/VCP may subtly alter the protein’s structure in a way that increases its propensity to aggregate when expressed in cells, generating an inactive or toxic protein species that then sequesters endogenous p97/VCP and/or its co-factors. These putative mechanisms could be consistent with IBMPFD mutations in p97/VCP causing a loss of p97/VCP’s function in the ubiquitin–proteasome and ERAD pathways or through a toxic gain of function related to aggregation of p97/VCP itself.

Although we have shown that IBMPFD mutant p97/VCPs affect ERAD, they may also perturb the enzyme’s interaction with other (non-ERAD) ubiquitinated proteins either directly (10,25) or indirectly (35,36). In addition, it is possible that the aggregation of IBMPFD mutant p97/VCP in cells affects ERAD and the ubiquitin–proteasome pathway independently of p97/VCP’s normal cell function. This mechanism has been suggested for other aggregate–prone proteins (35,37). Future development of specific assays for p97/VCP function in the ERAD and ubiquitin–proteasome pathway should allow us to determine precisely how IBMPFD mutations compromise p97/VCP function.

The rapid and striking effects caused by transient overexpression of high levels of IBMPFD mutant p97/VCP in cultured cells differ in time course from the changes seen in IBM/PFD patients. The natural course of IBM/PFD is insidious with an onset in the fourth or fifth decade of life (3). It may be that having normal levels of p97/VCP, in which 50% is mutant, cause disease in cells only after many years of abnormal levels of ubiquitinated proteins that are less than extreme than seen here following the high-level expression associated with transient transfections. Alternatively, the dysfunction associated with a patient’s complement of mutant p97/VCP may only manifest itself in response to specific stressors associated with aging muscle. Despite the fact that the effects seen in the current study, using transient transfection are much more rapid than those seen in patients, these cells do recapitulate many of the hallmark pathological features seen in IBM and IBM/PFD. These include aggregated protein containing ubiquitin and p97/VCP, as well as vacuoles containing proteinaceous debris. Interestingly, some ubiquitin-containing aggregates co-localize with sarcoplasmic reticulum membrane markers in IBM patient tissue (38).

IBMPFD and IBM are diseases of aggregated proteins similar to Alzheimer’s disease and Huntington’s disease (1,2). We propose that cells expressing IBM/PFD mutant p97/VCP will be a useful in vitro model of IBM pathology with close ties to disease pathogenesis. Future studies using cell culture and animal models of IBM/PFD mutations in p97/VCP should provide understanding of how skeletal muscle and other cells adapt to the accumulation of undegraded and aggregated proteins.

**MATERIALS AND METHODS**

**Cloning and DNA manipulation**

For mammalian expression of p97/VCP, mouse p97/VCP cDNA was PCR amplified and cloned into EGFPN1 expression vector (Clontech, Palo Alto, CA, USA). This construct fused GFP to the C-terminus of p97/VCP with a linker of VDPPVAT between the last residue of p97/VCP and the first residue of GFP. p97/VCP cloned into pet28a has been previously described and was used for bacterial expression (25). R155H, R95G and E578Q mutations were generated in the EGFPN1 or pet28 vector, using Quickchange mutagenesis (Stratagene). The sequences of all constructs were verified by nucleotide sequencing. p97/VCP-WT in pcDNA3.1myc/HisB (Invitrogen), p97/VCP-E578Q and p97/VCP-E305Q/E578Q in pet28a have been previously described (25).

**Cell culture, plasmid transfection and inclusion counting**

C2C12 cells were plated in 60 mm plates (for immunoblots) or on poly-l-lysine coated glass coverslips (for microscopy) in DMEM 10% fetal calf serum. Cells were transfected with Qiagen purified plasmid DNA, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s directions. For live cell counting, coverslips were rinsed once in phosphate buffered saline and inverted onto a microscope slide. Cells containing p97/VCP inclusions were counted using intrinsic GFP fluorescence. Cells in twenty randomly chosen fields from three independent transfection
experiments were counted for each condition 24 h after transfection. Qualitative analysis of cells expressing mutants for longer timepoints suggested that 24 h represented the peak in aggregate formation.

Purification, deep-etch freeze fracture EM and ATPase activity of bacterially expressed recombinant p97/VCP

Proteins were expressed in BL21(DE3) and purified as previously described (25). Peak protein fractions were snap-frozen in liquid nitrogen and protein concentrations were determined by Bradford assay, using BSA as a standard. Quick-freeze deep-etch EM of the p97/VCP molecules was performed as previously described (39). ATPase activities of p97/VCP, p97/VCP-R155H and p97/VCP-E305/E578Q were measured as described previously with four independent experiments for each enzyme (25).

Immunofluorescence, western blots and thin-section EM

Immunofluorescence of cultured cells was performed, using cells fixed in 4% paraformaldehyde/4% sucrose and permeabilized with 0.1% Triton X-100 or for p97/VCP-WT-myc co-transfected cells fixed and permeabilized in 100% ice-cold methanol for 15 min. The following antibodies were used: mouse monoclonal anti-PDI (Stressgen, Victoria, BC, Canada), rabbit anti-calnexin (Stressgen), rabbit anti-giantin (Covance, Richmond, CA, USA), rabbit anti-myc (Cell Signaling Technology, Beverly, MA, USA), mouse anti-CFTR (Upstate Biotechnology, Lake Placid, NY, USA). Secondary goat anti-mouse or goat anti-rabbit antibodies conjugated to Alexa 555 were from Molecular Probes (Eugene, OR, USA). Confocal microscopy was performed on a Radiance 2000 Bio-Rad microscope using 488 and 543 nm laser lines. All images were acquired with sequential scans, using LaserSharp 2000. Epifluorescence images were captured with a Leica Diaplan microscope, using a 63× 1.4-numerical aperture objective and a Zeiss AxioCam MRm. Images were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Transfected C2C12 lysates for fractionation were prepared by resuspending washed cells in 100 μL 25 mM Hepes (pH 7.4); 1 mM EDTA; 1 mM DTT; 0.5% Triton X-100 with protease inhibitor cocktail (Roche Diagnostics, Germany) and sonicated using a Branson Sonifier with 50% cycle with an output of six on ice for 5 min. The lysate was then spun at 16 000 g for 10 min and supernatant was collected. Pellets were resuspended in 1 × SDS Laemmli buffer. Supernatants were resuspended in an equal amount of 2 × SDS Laemmli buffer. For co-transfection experiments of p97 with CFTR, transfected cells from 35 mm dishes were scraped 36 h post-transfection, resuspended in 1 × Laemmli buffer, sonicated as earlier and heated at 37°C for 30 min prior to gel loading. Lysates were separated on a 10% SDS–PAGE gel, transferred to nitrocellulose and immunoblotted with an affinity-purified antibody (B5) or mouse anti-CFTR antibody. Immunoblots were developed by enhanced chemiluminescence with Supersignal reagent (Pierce, Rockford, IL, USA) as described (39). Equivalent amounts of protein loaded in each lane and confirmed using a mouse anti-actin antibody (Sigma, St Louis, MO, USA) control immunoblot.

For thin-section EM, transiently transfected cells were grown to 70% confluence in a 100 mm dish for 36 h. For sample preparation, trypsinized cells were collected, washed in phosphate-buffered saline, fixed in 2.5% glutaraldehyde in Na-cacodylate, embedded, sectioned and stained with uranyl acetate according to standard procedures.

Densitometric analysis

Autoradiographs of immunoblots from three independent experiments were scanned using an Epson 636 Expression Scanner. Densitometry of the undegraded CFTR band was analyzed using Scion Image analysis software (Scion Corporation, Frederick, ML, USA) and normalized to the actin loading standard, so as not to bias samples with more or less protein. For each experimental condition, the relative increase in undegraded CFTR from IBM/PD mutant co-transfected cells was determined by comparing it with the amount of undegraded CFTR in p97/VCP-WT-GFP co-transfectants from the same experiment. The average amount of undegraded CFTR present in p97/VCP-WT co-transfected cells was arbitrarily set to 1. The plotted histogram represents the average increase in undegraded CFTR from three independent experiments. Error bars represent standard deviation and P-values were determined by paired Student’s t-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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