Functional ATPase Activity of p97/Valosin-containing Protein (VCP) Is Required for the Quality Control of Endoplasmic Reticulum in Neuronally Differentiated Mammalian PC12 Cells*

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Abnormal protein accumulation and cell death with cytoplasmic vacuoles are hallmarks of several neurodegenerative disorders. We previously identified p97/valosin-containing protein (VCP), an AAA ATPase with two conserved ATPase domains (D1 and D2), as an interacting partner of the Machado-Joseph disease (MJD) protein with expanded polyglutamines that causes Machado-Joseph disease. To reveal its pathophysiological roles in neuronal cells, we focused on its ATPase activity. We constructed and characterized PC12 cells expressing wild-type p97/VCP and p97(K524A), a D2 domain mutant. The expression level, localization, and complex formation of both proteins were indistinguishable, but the ATPase activity of p97(K524A) was much lower than that of the wild type. p97(K524A) induced cytoplasmic vacuoles that stained with an endoplasmic reticulum (ER) marker, and accumulation of polyubiquitinated proteins in the nuclear and membrane but not cytoplasmic fractions was observed, together with the elevation of ER stress markers. These results show that p97/VCP is essential for degrading membrane-associated ubiquitinated proteins and that profound deficits in its ATPase activity severely affect ER quality control, leading to abnormal ER expansion and cell death. Excessive accumulation of misfolded proteins may inactivate p97/VCP in several neurodegenerative disorders, eventually leading to the neurodegeneration.

Various neurodegenerative disorders, including polyglutamine diseases, Parkinson’s disease, and amyotrophic lateral sclerosis, have distinct clinical symptoms, but they share several intracellular features such as accumulation of abnormal proteins or intracellular deposits of ubiquitinated proteins, formation of cytoplasmic vacuoles, and neural cell death (1). These observations suggest a potential link between neuronal degeneration and dysfunction of the protein degradation pathway via the ubiquitin-proteasome system (2, 3). Consistent with this, several inherited neurodegenerative disorders have been shown to be caused by mutations in genes whose products regulate the ubiquitin-proteasome system (4–6).

At least nine inherited neurodegenerative diseases, including Huntington’s disease and Machado-Joseph disease (MJD/SCA3),1 have been shown to be caused by the expansion of polyglutamine (poly-Q) in the proteins responsible for each disorder (1, 7, 8), and thus, this class of inherited neurodegenerative diseases is collectively called the polyglutamine diseases (1, 8, 9). We have identified p97/VCP, a member of the AAA ATPase family, as a binding partner of the MJD protein with the expanded poly-Q via biochemical purification and have shown that the binding is dependent on the length of the poly-Q; the longer the polyglutamine stretch, the stronger the interaction (10). By immunohistochemical analyses, p97/VCP was found to co-localize with poly-Q inclusions in cell culture and transgenic rat models overexpressing expanded poly-Qs of MJD protein origin (10).² Surprisingly, similar p97/VCP immunostaining was observed on Lewy bodies in brain samples of patients with sporadic Parkinson’s disease³ as well as dementia with Lewy bodies (10). p97/VCP immunostaining was also observed throughout aggresomes induced by proteasome inhibitor treatments in cultured neuronal cells, where many large vacuoles were concomitantly observed (10). Using our Drosophila poly-Q disease model (11), we performed a genetic screen to search for enhancers and suppressors of the eye degeneration phenotypes caused by the poly-Q expression and identified ter94, the Drosophila p97/VCP, as a modulator of poly-Q-induced eye degenerations (11). These observations collectively indicate that p97/VCP is a key molecule in the pathway of neuronal cell death induced by expanded poly-Q and probably other misfolded proteins as well.

p97/VCP is one of the most abundant intracellular proteins, and it has been shown to be involved in postmitotic processes of membrane fusions of endoplasmic reticulum (ER), Golgi apparatus, and nuclear envelopes (12–15). In these processes, p97/VCP differentially utilizes its partners such as p47 and the Ufd1/Npl4 complex. Recently, p97/VCP has also been shown to bind directly to polyubiquitinated proteins in vitro (16) and to be involved in the ER-associated protein degradation system (ERAD) via interaction with Ufd1 and Npl4 in yeast and permeabilized astrocytoma cells (17–20).

In this study, we demonstrate that the D2 ATPase domain represents the major ATPase activity in p97/VCP and is essential for p97/VCP function, especially to maintain ER integrity

1 The abbreviations used are: MJD, Machado-Joseph disease; VCP, valosin-containing protein; poly-Q, polyglutamine; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; CFTR, cystic fibrosis transmembrane conductance regulator protein; GFP, green fluorescent protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; EYFP, enhanced YFP.
2 S. Hori, K. Inoue, and A. Kakizuka, unpublished observations.
3 M. Takanashi, N. Hattori, Y. Mizuno, and A. Kakizuka, manuscript in preparation.

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The affinity-purified rabbit polyclonal anti-p97 antibodies were incubated in the same buffer without low fat milk. Antibody and Plasmons—The affinity-purified rabbit polyclonal anti-p97/VCP antibody was described previously (10). The positions of exogenous and endogenous p97s are marked as p97-GFP or p97(K524A)-GFP and p97, respectively. After removal of tetracycline, p97-GFP and p97(K524A)-GFP were expressed comparably, and their intracellular localizations were indistinguishable from that of endogenous p97/VCP.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Lines—**PC12 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (low glucose) supplemented with 10% fetal calf serum and 5% horse serum. TV and TmV cells were maintained following a standard procedure as described previously (21) and were maintained in the same media for PC12 with the addition of the tetracycline (0.5 μg/ml). HER293 cells were grown in Dulbecco’s modified Eagle’s medium (high glucose) with 10% fetal calf serum. Western blot using anti-p97 antibody. The following antibodies were used in this study: monoclonal anti-ubiquitin (anti-Ub; Chemicon); monoclonal anti-GFP (Roche Diagnostics); goat polyclonal anti-CTFR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis. A GFP-CFTR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis. A GFP-CFTR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis. A GFP-CFTR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis. A GFP-CFTR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis. A GFP-CFTR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis. A GFP-CFTR (C-19); and rabbit polyclonal anti-GADD153 (CHOP) (Santa Cruz Biotechnology, Inc.). Mouse p97/VCP cDNA was subcloned into the pcIX vector (9, 21, 22). p97(K524A)-expressing plasmids were constructed by site-directed mutagenesis.

**Expression and Purification of p97/VCP and Its Cofactor—**Mouse p97/VCP and p97(K524A) cDNAs were subcloned into the pEYFP vector (Clontech). cDNAs encoding His-YFP-p97s were subcloned into a baculovirus expression vector. Recombinant His-YFP-p97s were expressed in insect sf-9 cells after transfection with CELFFECTIN Reagent (Invitrogen). Cells were lysed in a lysis buffer (400 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 1 mM ATP, 5 mM β-mercaptoethanol, 100 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 20 mM benzamidine, and a protease inhibitor mixture (Nakarai Tesque), and the lysates were loaded onto nickel-chelated Hitrap chelating columns (Amersham Biosciences) and washed with a buffer containing 500 mM NaCl, 50 mM potassium phosphate (pH 7.8), and 50 mM imidazole. The recombinant proteins were eluted with a 50–500 mM imidazole gradient, concentrated by Centriprep (Millipore), and kept in storage buffer (150 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, 40% glycerol) after passing through a PD-10 column (Amersham Biosciences). The entire coding regions of p47, Ufd1, and Npl4 cDNAs were amplified from a human brain lysate via reverse transcriptase-PCR and cloned into pBlueScriptII KS (+) (Stratagene), and their sequences were confirmed. These cDNAs and MJDQ79 cDNA (21) were subcloned into the pGEX vectors (Amersham Biosciences). Recombinant GST-fused proteins were expressed in Escherichia coli, purified with glutathione-Sepharose (Amersham Biosciences), and eluted with 25 mM glutathione, and the buffers were exchanged into PBS with dialyses.

**Density Gradient and Pull-down Experiments—**HeLa, TV, and TmV cells were harvested at the indicated time periods, washed with ice-cold PBS, and lysed in Triton buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor mixture (complete™). Cell debris was removed by centrifugation at 12,000 × g for 10 min. The supernatants were loaded on a glycerol gradient (10–40%) and centrifuged in a SW41 (Beckman) rotor for 12 h at 36,000 rpm. For pull-down experiments, cell lysates (100 μg) were mixed with 2 μg of GST-fusion proteins and rotated overnight, and then glutathione beads were added with rotation for another 4 h. Beads were washed with Triton buffer. Bound proteins were eluted with sample buffer and analyzed by SDS-PAGE (24–26).

**Protein Detection in Western Blot—**Blocking and primary antibody incubations were performed in Tris-buffered saline plus 0.02% Tween 20 and 5% low fat milk. Horseradish peroxidase-conjugated secondary antibodies were incubated in the same buffer without low fat milk. Proteins were visualized and quantified using the ECL detection kit.
Fig. 2. Complex formations of p97(K524A) with endogenous p97/VCP and its partners. A, homo-hexamer formation of exogenous p97-GFPs with endogenous p97/VCP. Cell lysates were prepared from control HeLa cells and TV and TmV cells 48 h after removal of tetracycline. Cell lysates were separated on 10–40% glycerol density gradients and collected in 20 fractions using the density gradient fractionator after centrifugation. Pellets were directly resolved in SDS buffer and were loaded in the same SDS-PAGE for Western analysis (Bottom). Positions of co-migrated molecular mass markers (marker) are indicated below the panels. B, heteromeric complex formation of exogenous p97-GFPs with p97 partners. Cell lysates from TV and TmV cells 48 h after removal of tetracycline were mixed with GST alone or GST-fused p97 partners, which are described above the panels, pulled down with glutathione beads by centrifugation. The pellets were examined by Western analyses using an anti-GST or an anti-p97 antibody.

(Amersham Biosciences) and a luminescence image analyzer (LAS-1000 PLUS, Fuji Film) (24–26).

Subcellular Fractionation and Immunoprecipitation—TV and TmV cells were harvested, chilled on ice, washed with PBS, and pelleted at low centrifugation. Cell pellets were suspended in homogenizing buffer (0.25 M sucrose, 10 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor mixture, passed through 22-gauge needle, and homogenized using a Dounce homogenizer (Wheaton) for 12 strokes. Nuclear, membrane, and cytosolic fractions were separated by sequential centrifugation at 700 × g for 20 min followed by centrifugation at 100,000 × g for 60 min. For immunoprecipitation, samples were solubilized with 1% Triton X-100. Insoluble portions were removed by centrifugation at 12,000 × g for 10 min. The supernatant was mixed with an anti-GFP antibody and rotated at 4 °C overnight after addition of protein G-Sepharose beads (Amersham Biosciences). The beads were washed three times with Triton buffer. Proteins were eluted with sample buffer and analyzed by SDS-PAGE (24–26).

FACS Analysis—HEK293 cells were transfected with a GFP-CFPTR(ΔF508) expression vector, harvested, washed with PBS, fixed by 70% EtOH on ice for 20 min, and washed with PBS containing 1% bovine serum albumin, and GFP intensities and cell numbers were measured by FACS scanning (BD Biosciences).

Measurement of ATPase Activities—The ATPase activities of p97/VCP were assayed by an enzyme coupling method, as described (27). Briefly, the rate of ATP hydrolysis was calculated from the linear phase of the decrease in the absorbance at 340 nm of NADH at 37 °C in an assay buffer (50 mM Tris-HCl (pH 9.0), 150 mM NaCl, 2 mM MgSO4, 3 mM phosphoenolpyruvate, 0.25 mM NADH, and 2 mM ATP) supplemented with enzymes (1.5 units of pyruvate kinase and 1.0 unit of lactate dehydrogenase).

RESULTS

Construction of PC12 Cells Stably Expressing p97/VCP and Its Mutant—To elucidate the pathophysiological roles of p97/VCP, we constructed stable neuronal PC12 cells expressing wild-type and mutant p97/VCP with C-terminal-tagged GFP under the control of the tet-off promoter and compared the phenotypes of these cells after tetracycline removal. PC12 cells are a well-characterized cell line with the ability to differentiate into postmitotic neuron-like cells by the simple addition of nerve growth factor. Before this experiment, we made several mutants in which the conserved lysines (251 and 524) or glutamates (305 and 578) were substituted with alanine or glutamine residues, respectively, resulting in the following mutants: p97(K524A), p97(K524A), p97(E578Q), and p97(E578Q). These amino acids are essential residues in the Walker A or B motif of the ATP binding domain. We selected the p97(K524A) mutant for this study because it induced cytoplasmic vacuoles and cell death most effectively in differentiated PC12 cells, when expressed transiently; the p97(E578Q) mutant had a similar but weaker phenotype than p97(K524A), and the others had much more subtle phenotypes. Among the several sublines, we selected cells with the highest expression levels of wild-type p97-GFP and p97(K524A)-GFP after tetracycline removal, and these cells were named TV and TmV cells, respectively (Fig. 1).

Expression and Localization of Induced p97-GFPs in PC12 Cells—We next compared the protein levels between p97-GFP and p97(K524A)-GFP in TV and TmV cells, respectively. The expression levels of both proteins appeared equivalent and were comparable with the endogenous p97/VCP protein 24 and 48 h after the removal of tetracycline from the medium (Fig. 1, A (right panels) and B). Localization of both proteins was observed throughout the cells (Fig. 1A, left panels), a pattern indistinguishable from that of endogenous p97/VCP. Consistent with this, the subcellular fractionation analysis also showed that both p97-GFP and p97(K524A)-GFP expression were comparable with and behave indistinguishably from the endogenous p97/VCP protein (Fig. 1B). In the presence of tetracycline, both cells grew well and responded equally to nerve growth factor with formation of neurite-like structures (data not shown). However, we found clear differences in the morphology between TV and TmV cells after removal of tetracycline from the medium. TV cells maintained a healthy appearance and were indistinguishable from the parental PC12 cells with numerous neurite outgrowth, whereas TmV cells appeared sick and lost the ability to extend neuritis and formed many cytoplasmic vacuoles instead (Fig. 1A, middle panels, and see below), followed by cell death. This cell death was not inhibited by a calpain inhibitor or several caspase inhibitors, e.g. Z-VAL-FMK (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) and Ac-DEVD-CHO (Ac-Asp-Glu-Val-Ala dehydroace) (data not shown).

Homo- and Heteromeric Complex Formation and ATPase Activity of p97(K524A) Mutant—Homo-oligomeric ring structure is the most characteristic feature of AAA ATPase, which has been shown to be required for representing the maximum ATPase activity. To examine whether p97-GFP and p97(K524A)-GFP can form their hexameric structure in PC12 cells, lysates of TV and TmV cells were first fractionated by the density gradient method, and then each fraction was analyzed...
We also expressed His-YFP-tagged p97/VCP and p97 (K524A) proteins in the baculovirus system and then affinity-purified these proteins using a nickel column (Fig. 3A, lanes 1 and 2). Small amounts of endogenous p97/VCP were co-purified with both recombinant proteins (Fig. 3A, lanes 1 and 2, Coomassie Brilliant Blue staining, and lanes 3 and 4, immunoblot). The recombinant p97/VCP complex showed more than 23-fold stronger ATPase activity when compared with the recombinant p97(K524A) complex (Fig. 3B). These results clearly show that alanine substitution of lysine 524 abolished most of the ATPase activity of p97/VCP and thus indicate that the second ATPase domain represents the major ATPase activity in p97/VCP.

Regardless of the change in the ATPase activity, the GST pull-down experiments showed that the p97-GFP and p97(K524A)-GFP hexameric complexes co-precipitated with GST-fused p47, Ufd1, Npl4, and MJD (Fig. 2B). Both p97-GFP and p97(K524A)-GFP complexes were also immunoprecipitated with endogenous p47, Ufd1, and Npl4 using anti-p47, anti-Ufd1, and anti-Npl4 antibodies, respectively (data not shown). These results indicate that p97/VCP ATPase activity is dispensable for its interaction with known partners, e.g. p47, Ufd1, Npl4, and MJD.

p97(K524A) Induces ER Stress and ER Expansion—As mentioned above, 24 h after expression of p97(K524A), many large vacuole-like membrane compartments appeared in the cytoplasmic spaces of TmV cells followed by cell death. To investigate the origin of these strange organelles, we examined which organelle markers, e.g. mitochondria, Golgi apparatus, lysosome, endosome, and endoplasmic reticulum (ER) markers, could stain them. Among those tested, only CFP-ER, an ER-residing marker protein that has the ER localization signal of calreticulin and the KDEL motif, gave clear co-localization with the vacuoles (Fig. 4, A–C). This result was consistent with our previous electron microscopy results that revealed ribosome-like structures scattered on the p97(K524A)-induced vacuole membranes (10). Treatment with PSI, a proteasome inhibitor, also induced similar CFP-ER-positive vacuoles and cell death even in parental PC12 cells (Fig. 4, C and D).

Given that these vacuoles were of ER origin, it would be reasonable to speculate that ER stress might be induced in TmV cells. We thus examined the expression of GRP78 mRNA, an ER stress marker. Significant up-regulation of GRP78 mRNA was observed in TmV but not in TV cells 24 h after removal of tetracycline (Fig. 5A, lanes 1 and 4). Similar levels of GRP78 mRNA were also induced after treatment with tunicamycin (11), a known ER stress inducer that functions through inhibiting protein N-glycosylation (Fig. 5A, lanes 1 and 2). CHOP protein (28), another ER stress marker, was also clearly induced in TmV but not in TV cells 1–2 days after removal of tetracycline (Fig. 5B). These results indicate that the loss or severe decrease of p97/VCP ATPase activity results in ER stress and abnormal ER expansion.

p97/VCP Is Involved in the ER Quality Control System via Membrane Protein Degradation—As mentioned above, cytoplasmic vacuoles of ER origin were also induced by the addition of proteasome inhibitors in the culture medium of PC12 cells (Fig. 4, right panel). It has been shown that approximately one-third of newly synthesized proteins in the cells are not appropriately folded, and these misfolded proteins were degraded mainly via the ubiquitin-proteasome system. We thus next examined a potential link between protein degradation and the p97/VCP ATPase activity and found that expression of p97(K524A)-GFP enhanced the accumulation of ubiquitinated and likely misfolded proteins in TmV cells (Fig. 6, A lanes 4–6, 10–12, and 16–18) and B). However, the accumulation of ubiquitinated proteins is prominent in the nuclear (lanes 11 and 12).

Fig. 3. ATPase activities of wild-type p97/VCP and p97(K524A). A, Recombinant wild-type p97/VCP and p97(K524A) were produced with the baculovirus-mediated expression system. Coomassie Brilliant Blue staining (lanes 1 and 2) and Western analyses (lanes 3 and 4) of purified recombinant proteins using the indicated antibodies are shown. YFP is a derivative of GFP that is recognized by anti-GFP antibodies. WT, wild-type. Marker, molecular mass markers. B, ATPase activities of recombinant p97s purified from insect cells. ATPase activities were measured at various concentrations of p97/VCP using an enzyme-coupling assay system at 37 °C (see details in “Experimental Procedures”). Three independent measurements of wild-type p97/VCP (open circles) and p97(K524A) (open squares) are shown.
and membrane fractions (lanes 17 and 18) but not in the cytoplasmic fraction (lanes 23 and 24), indicating that loss or decrease of the p97/VCP ATPase activity did not inhibit cytoplasmic protein degradation where proteasomes are functional. Then, how is p97/VCP involved in the protein degradation in places other than the cytoplasm?

We performed immunoprecipitation analysis using anti-GFP antibody (Fig. 6C). Membrane and cytosolic fractions of TV and TmV cells were resolved by 1% TritonX-100 and clarified by centrifugations. The resultant soluble proteins were immunoprecipitated with anti-GFP antibody (Fig. 6C) or a non-related anti-FLAG antibody as a control (data not shown), and precipitates were resolved by SDS-PAGE and detected by an anti-ubiquitin antibody via Western blot analysis. The results showed that p97(K524A)-GFP co-precipitated more polyubiquitinated proteins from the membrane but not the cytoplasmic fractions than p97-GFP. The precipitates from the membrane fraction contained endogenous Ufd1, Npl4, and p47 (data not shown). It is noteworthy that the ER quality control system senses misfolded proteins and refolds them with chaperones or degrades them by the ER-associated degradation (ERAD), in which misfolded proteins are translocated into the cytoplasm through the Sec61 translocon and then degraded by the ubiquitin-proteasome system in the cytoplasm. Consistent with recent reports (18, 29, 30), these results support the idea that p97/VCP functions in the ERAD system, especially in pulling out misfolded membrane proteins into the cytoplasm.

We next examined whether the degradation of misfolded membrane proteins was inhibited in cells expressing p97(K524A) by using CFTR(F508) as a model substrate.
which is a well known substrate of ERAD (23). By microscopy and FACS analyses, we could observe more GFP-CFTR(H9004F508) aggregates in cells expressing p97(K524A) than in cells expressing wild-type p97/VCP or p97(K251A) (Fig. 7A). Furthermore, it was evident that more GFP-CFTR as ubiquiti-nated high molecular weight forms (31) accumulated in cells expressing p97(K524A) when compared with cells expressing wild-type p97/VCP or p97(K251A) (Fig. 7B). These results collectively indicate that the ATPase activity of p97/VCP is essential in the ER quality control system and that its severe dys-function leads to ER stress and cell death.

**DISCUSSION**

p97/VCP belongs to the AAA class ATPase with two ATPase domains (D1 and D2) and has been shown to take a homo-hexameric structure in normal cellular conditions (32). p97/VCP is known to perform a variety of cellular functions through interaction with its function-specific partners, e.g. p47, Ufd1, and Npl4 (see Introduction). It is noteworthy that p97/VCP was affinity-purified as an interacting protein with MJD79 (10), the MJD gene product with an expanded 79 repeat of polyglutaminates that causes Machado-Joseph disease (7, 9, 21), and that p97/VCP is believed to be a sensor that detects abnormal protein accumulation in the cell (10). Interestingly, expression of p97(K524A), in which the conserved 524th lysine in the D2 domain was replaced by alanine, induced phenotypes reminiscent of those observed commonly in human neurodegenerative disorders, such as cytoplasmic vacuoles and cell death (Ref. 10 and see below).

In this study, to elucidate the pathophysiological roles of p97/VCP, we characterized the p97(K524A) protein and neuro-nally differentiated mammalian cells expressing p97(K524A). Recombinant baculovirally produced p97(K524A) had very weak ATPase activity (Fig. 3B) but retained the ability to interact with all partners tested, e.g. p47, Ufd1, Npl4, and MJD proteins, indicating that ATPase activity is not necessary for p97/VCP to interact with its partners (Fig. 2B). Furthermore, p97(K524A) was able to be incorporated in the hexameric complex as effectively as the wild-type protein (Figs. 2A and 6C), also indicating that ATPase activity is not necessary for the hexameric structure formation of p97/VCP.

In the cells expressing p97(K524A), we observed many large cytoplasmic vacuoles followed by cell death; these phenotypes were observed in transient transfections (10). It is notable that this cell death could not be blocked by caspase inhibitors (data not shown). The vacuoles were stained by CFP-ER, an ER marker-fused cyan fluorescent protein (Fig. 4), and ribosome-like density was observed on the membranes by electron microscopy (10), indicating an ER origin. Consistent with these observations, in the vacuolated cells, typical ER stress markers (GRP78 mRNA and CHOP protein) were induced at levels...
comparable with those observed by treatment with tunicamycin, a well known ER stress inducer. Tunicamycin further enlarged these vacuoles, whereas the addition of nocodazole and Brefeldin A, known inhibitors of ER-Golgi vesicular transport, (33) did not have any significant effects. These results suggest that the observed ER expansion does not depend on the vesicular traffic between ER and Golgi but is likely to depend on the strength of the ER stress.

Cellular fractionation experiments revealed that ubiquitinated proteins accumulated not only in the membrane but also in the nuclear fractions of cells expressing p97(K524A) but not wild-type p97/VCP. Moreover, we found that p97(K524A) remained bound to ubiquitinated proteins (Fig. 6). p97/VCP, p97(K251A), or p97(K524A). Twenty-four h after transfection, aggregates were analyzed by fluorescence microscopy (upper panels) and FACS analysis (lower panels). In the FACS analysis, the intensities of GFP fluorescence are presented on the x axis and gated at M1, M2, M3, and M4, as shown in the panels. The ratios in cell numbers of M2, M3, and M4 to total cell numbers are shown in parentheses. Similar M1 fluorescence was also observed in non-transfected cells (data not shown). Note the increased cell ratios in M3 and M4 in the p97(K524A) transfection. As shown in E, total cell lysates from cells 24 h after transfection in the conditions described in panel A were analyzed by Western blot using an anti-CFTR antibody. Cell lysate from non-transfected cells gave no signals (lane 4). Note the increased accumulation of high molecular mass species (HMM) of GFP-CFTR(ΔF508) in the p97(K524A) transfection.

These results fit well with the model in which p97/Ufd1/Npl4 is a protein complex that is involved in pulling out misfolded proteins from the ER (17–20) and the nucleus (as shown in this study). The former is a well known phenomenon called ER-associated protein degradation (ERAD). In the first step of this model in ERAD, p97/VCP recognizes its membrane-associated substrates that were ubiquitinated; this is coincident with the report that p97/VCP binds multi-ubiquitin chains (16). In the following step, using its ATPase activity, p97/VCP appears to extract and then to release the substrates into the cytosplasmic space, where proteasomes can degrade them. Thus, a profound decrease in p97/VCP ATPase activity, as typically observed with p97(K524A), is expected to block ERAD. Indeed, CFTR(ΔF508) aggregates were enhanced by p97(K524A) expression: CFTR(ΔF508) is a well known substrate of ERAD (Fig. 7). p97/VCP may also perform chaperone-like functions using its ATPase activity to unfold substrates, as reported in the case of VAT, the Archaebacteria homologue of p97/VCP (35). Further ubiquitination might be important for unfolding the substrates by inhibiting their backward transport into the ER or nucleus.

This model also provides an explanation for the abnormal ER expansion observed in cells expressing p97(K524A). Due to ERAD inhibition, a substantial increase of degradation-des-tined proteins and/or accumulation of proteins such as ER chaperones, ERAD components, lipid synthesis enzymes, and so forth, induced in response to the continued presence of unfolded proteins (36), may physically enlarge the ER.

Since p97/VCP functions as a hexamer, seven different ATPase activities could be theoretically created in one complex with p97(K524A), from zero to six, which could in turn create several varying degrees of ERAD inhibition. Indeed, vacuoles and cell death induced by p97(K524A) were inhibited by the expression of wild-type p97/VCP (10). Furthermore, considering the high level of expression of endogenous p97/VCP, the overall ATPase activities of p97/VCP in the cells are expected to change dramatically as a consequence of the p97(K524A) expression levels. In recent studies, the accumulation of misfolded proteins has been shown to impair the proteasome activity in several experimental models of neurodegenerative disorders, including polyglutamine diseases (2, 3, 37). Our studies showed that mild inhibition of proteasomes by inhibitory drugs gives rise to phenotypes resembling those observed.
in cells expressing p97(K524A) or those observed via the pathological and biochemical analysis on several neurodegenerative disorders (37–40). These results suggest that accumulation of misfolded proteins inhibits not only the proteasome activity but also the ATPase activity of p97/VCP to different degrees, which in turn contributes to the inhibition of the ERAD system, also in different degrees. Given that the above situation exists, what is the significance of ERAD system inhibition? Cells are continuously burdened by having to degrade a large quantity of misfolded proteins. Approximately 30% of the newly created proteins are reportedly misfolded (41), and therefore cells need to monitor the amount of misfolded proteins present within them and respond to maintain this amount at a constant level. In this mechanism, we propose the following model. First, accumulation of misfolded proteins in the cytoplasm is sensed by p97/VCP either via direct binding or via ubiquitin-mediated binding. Second, proportional to the amount of p97/VCP bound by misfolded proteins, its ATPase activity is reduced. The more p97/VCP units that are occupied by misfolded proteins in the hexameric complex, which reflects the increase of misfolded proteins, its ATPase activity is reduced. The more misfolded proteins, its ATPase activity is reduced. The more...
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