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Dominant-negative effect of mutant valosin-containing protein in aggresome formation

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Abstract Lewy bodies (LBs) are the pathologic hallmark of Parkinson's disease. Recent studies revealed that LBs exhibit several morphologic and molecular similarities to aggresomes. Aggresomes are perinuclear aggregates representing intracellular deposits of misfolded proteins. Recently, valosin-containing protein (VCP) was one of the components of LBs, suggesting its involvement in LB formation. Here, we showed the localization of VCP in aggresomes induced by a proteasome inhibitor in cultured cells. Cells overexpressing mutant VCP (K524M: D2) showed reduced aggresome formation relative to those overexpressing wild-type and mutant (K251M: D1) VCPs. Our findings suggest that the D2 domain is involved in aggresome formation.

Keywords: Valosin-containing protein; Parkinson's disease; Lewy body; Aggresome

1. Introduction

Parkinson's disease (PD) is characterized histopathologically by the relatively selective loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies (LBs) [1]. Recent studies suggest that LBs are related to aggresomes [2]. The latter are formed upon exhaustion of neuronal cell machinery responsible for the degradation of misfolded proteins [3]. Furthermore, aggresomes are formed at the microtubule (MT)-organizing center (MTOC) and are defined as pericentriolar membrane-free cytoplasmic inclusions that contain misfolded, ubiquitinated proteins ensheathed in a cage of the intermediate filament (IF) protein, vimentin [3,4].

Valosin-containing protein (VCP) is a 97-kDa protein and a member of type II ATPases associated with a variety of cellular activities (AAA), which are characterized by the presence of two conserved ATPase domains, also called AAA domains [5]. Recent studies revealed that VCP acts as a molecular chaperone in many apparently unrelated cellular activities [6]. Among these activities, VCP recognizes misfolded proteins such as polyglutamine [7]. Indeed, VCP is recognized in nuclear inclusion bodies of polyglutamine diseases [8]. Moreover, VCP is also recognized in LBs of PD [8,9].

Based on the above background, we postulated that VCP is involved in the formation of aggresomes. To test this, we examined the induction of VCP in aggresomes of cells treated with a proteasome inhibitor, MG132, and the role of VCP in aggresome formation.

2. Materials and methods

2.1. Plasmids and antibodies

We constructed pUHD10-3/VCP^{WT}-Myc, pCMV/FLAG-6c-VCP^{WT}, and pCMV/FLAG-6c-VCP$^{K251M/K524M}$ for this experiment. pUHD10-3/VCP^{WT}-Myc was digested and inserted into the *Xho*I site of the pcDNA3.1 vector (Invitrogen). pcDNA3.1/FLAG-VCP^{WT} was prepared by polymerase chain reaction (PCR) using appropriately designed primers with restriction site (*Bam*HI). The PCR product was inserted into the pcDNA3.1 vector. pcDNA3.1/FLAG-VCP^{K251M}, FLAG-VCP^{K524M} were prepared by using QuikChange Site-Directed Mutagenesis Kit (Stratagene). These point mutants lack two ATPases activity domains such as D1 and D2 [8]. pcDNA3.1/α-synuclein^{WT} and pcDNA3.1/FLAG-IκBα^{WT} were kind gifts from Drs. Suzuki and Chiba (The Tokyo Metropolitan Institute of Medical Science, Tokyo). We prepared FLAG-tagged α-synuclein to examine the interaction between VCP and α-synuclein.

The following antibodies were used in the present study; anti-VCP polyclonal antibody (Santa Cruz), anti-ubiquitin monoclonal antibody (Chemicon), anti-FLAG polyclonal antibody (Affiniti), anti-FLAG-HRP (Affiniti), anti-Myc monoclonal antibody (Santa Cruz), anti-vimentin monoclonal antibody (Sigma), anti-β actin monoclonal antibody (Sigma), anti-γ-tubulin monoclonal antibody (Sigma), and anti-Hsp70 antibody (BD Transduction Laboratories).

2.2. Cell culture and transfection

Kidney cell lines, HEK293 cells were grown in Dulbeccos modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Confluent cells were transfected with 9 μg Myc-vector, VCP^{WT}-Myc, 5 μg FLAG-α-synuclein and 2 μg FLAG-IκBα. At 24 h after transfection, the cells were lysed with 500 μl lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 1.0% nonidet-P40, 10% glycerol, 1 M dithiothreitol) and protease inhibitor cocktail; Complete Mini. The lysate was then centrifuged at 17 000 × *g* for 15 min at 4 °C, and then 30 μl volume of the supernatant was used as the ''lysate'' for SDS–PAGE, while 450 μl volume of the supernatant was used for immunoprecipitation. For immunoprecipitation, 2 μg anti-Myc antibody was added to each 450 μl of the supernatant and the mixture was rotated for 3 h at 4 °C, then centrifuged at 17 000 × *g* for 15 min. The supernatants were mixed with 20 μl protein G-Sepharose (Amersham Biosciences), rotated for 3 h at 4 °C, then

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centrifuged at 1800 × *g* for 5 min, washed three times and then mixed with 30 μl of the sample buffer. The samples were separated by SDS–PAGE (10–20% gradient gel) and transferred onto a PVDF membrane. Finally, detection was performed with anti-FLAG-HRP antibody (1:2000) and anti-Myc monoclonal antibody (1:2000).

2.3. Cell culture and immunological analysis

SH-SY5Y neuroblastoma cell and HEK 293 cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Confluent cells were treated with 10 μM MG132 (Sigma), proteasome inhibitor and dimethyl sulfoxide (DMSO; Sigma) (for control) for 0, 4, or 8 h. The cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 1.0% nonidet-P40, 10% glycerol and cocktail: Complete Mini; Roche). The lysate was then centrifuged at 17000 × *g* for 15 min at 4 °C. Next, the supernatant was used as the "crude". We considered the presence of aggregation in the pellet fraction and accordingly the pellet was solubilized with a mixture of 6 M urea, 50 mM Tris–HCl [pH 7.5], and 5 mM 2-mercaptoethanol, and sonicated. We then added the sample buffer to the sonicated sample as the "pellet". The whole lysates were prepared using the same method. The samples were separated by SDS–PAGE and transferred onto a PVDF membrane. Finally, detection was performed with VCP polyclonal and ubiquitin monoclonal antibodies.

2.4. Immunohistochemistry

After growing on 35-mm dishes (with glass coverslips), SH-SY5Y or HEK293 cells were treated with 10 μM MG132 or DMSO for 24 h. The cells were fixed in 4% paraformaldehyde in PBS for 30 min and permealized with Triton-X 100 for 20 min. Then, the cells were blocked overnight at 4 °C with 4% normal goat serum in PBS, incubated overnight with anti-VCP and anti-ubiquitin, anti-vimentin, anti-γ-tubulin, anti-Hsp70, and anti-FLAG antibodies in each case, washed with 0.01% Triton-X 100, and incubated for 30 min with Alexa 543 nm anti-mouse antibody and FITC 488 nm anti-rabbit antibody. The coverslips were washed and mounted on one vectashield. Fluorescence images were obtained using a fluorescence microscope.

2.5. Cell viability

HEK293 cells were grown under the same conditions. Confluent cells were transfected with 5 μg of FLAG-vector, FLAG-VCP^{WT}, FLAG-VCP^{K251M}, FLAG-VCP^{K524M}, or FLAG-VCP$^{K251M/K524M}$. The next day, the cells were transferred to 96-well dishes. After 1 h, the cells were treated with 10 μM MG132 or DMSO, and the cell viability was analyzed the next day. We used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay kit (Dojindo). We repeated the same experiments four times. To confirm the efficiencies of expression level, transfected cells were lysed with lysis buffer and centrifuged. We then added the sample buffer to the supernatant samples and applied them on the SDS–PAGE. In addition, we performed Western blotting with anti-FLAG, anti-VCP and anti-β-actin antibodies.

3. Results and discussion

Recent studies showed that cells treated with MG132 form aggregates that resemble LBs [10]. We first investigated whether VCP exists in such aggregates. As shown in Fig. 1, VCP was found in an aggregate formed under MG132. We also found that VCP levels were increased in the insoluble fraction, similar to polyubiquitinated proteins under MG132 condition, but not DMSO (Fig. 2). This finding indicates that these aggresomes were detergent insoluble and that VCP and ubiquitinated proteins were components of this fraction. Under this condition, the supernatant VCP fraction did not decrease at the same time, suggesting upregulation of VCP in the detergent-insoluble fraction. On the other hand, no increase in the whole VCP fraction was detected, suggesting that the amounts of supernatant fractions were larger than the insoluble fractions.

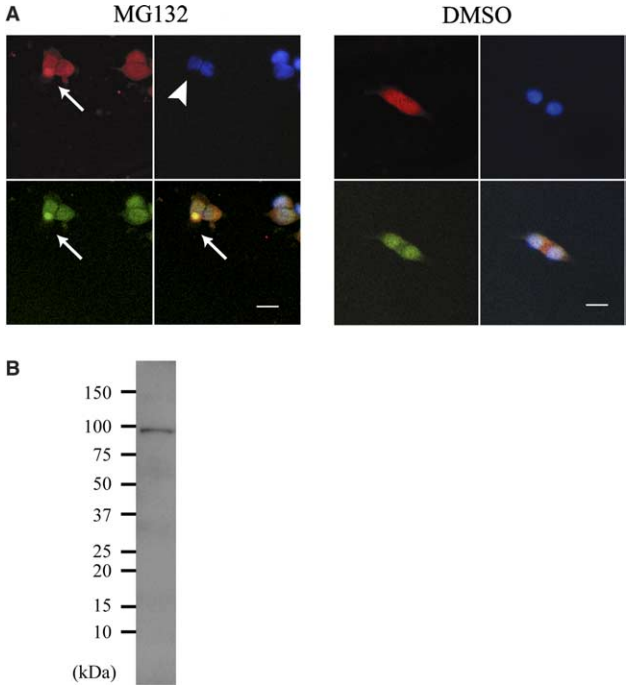

Fig. 1. Subcellular localization of endogenous VCP and ubiquitin in SH-SY5Y cells treated or untreated with a proteasome inhibitor. SH-SY5Y cells were treated with 10 μM MG132 (A) or DMSO (B) for 24 h. Cells were stained with anti-VCP and anti-ubiquitin antibodies. Nuclei were stained with DAPI (blue). FITC (green) and Alexa (red) correspond to VCP and ubiquitin, respectively. After treatment with MG132, both VCP and ubiquitin showed perinuclear accumulation and colocalization and appeared as clear protein aggregates (arrows). Nuclear torsion was observed (arrowhead). Scale bar = 20 μm.

In the next step, we investigated the localization of endogenous VCP in aggresomes. VCP formed a single large perinuclear aggresome-like structure and was co-localized with ubiquitin. Nuclear torsion was also noted (Fig. 1). Then we characterized the structure of these aggresomes to determine whether it is similar to that of typical aggresomes [3]. As shown in Fig. 3A, VCP-positive aggresomes were surrounded by vimentin in MG132-treated cells. Aggregates of misfolded proteins that escape degradation are targeted and accumulate in the MTOC. Subsequently, aggresomes are formed in the MTOC [3]. First, we showed that VCP-positive aggresomes co-localized with γ-tubulin, a marker of MTOC (Fig. 3B). Second, aggresomes are also abundant in chaperones such as Hsp70 [11]. We observed that Hsp70 co-localized with VCP in such aggresomes (Fig. 3C). Finally, we investigated the effect of inhibition of microtubule dynamics using an anti-mitotic agent, nocodazole, on the formation of these aggresomes. Co-incubation of cells with 10 μM nocodazole and MG132 resulted in inhibition of aggresome formation as evident in VCP and vimentin staining (Fig. 3D). These results indicate that the VCP-positive aggregates in SH-SY5Y cells are typical aggresomes.

We next examined the involvement of VCP in aggresome formation. We prepared FLAG-vector, FLAG-VCP^{WT}, FLAG-VCP^{K251M}, FLAG-VCP^{K524M}, and FLAG-VCP$^{K251M/K524M}$. The latter three vectors encode proteins lacking the function of two ATPase domains, D1 and D2, respectively. Considering the transfection efficiency, we used