Roles of VCP in human neurodegenerative disorders

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Abstract
Abnormal protein aggregates are commonly observed in affected neurons in many neurodegenerative disorders. We have reported that VCP (valosin-containing protein) co-localizes with protein aggregates in neurons of patients and in cultured cells expressing diseased proteins. However, the significance of such co-localization remains to be elucidated. In the present paper, I discuss the involvement of VCP in the processes of both the formation and re-solubilization of abnormal protein aggregates. In the study, VCP recognized and accumulated on to pre-formed protein aggregates created by proteasome inhibition. VCP knockdown or expression of dominant-negative VCP both significantly delayed the elimination of ubiquitin-positive aggregates. VCP was also involved in the clearance of pre-formed polyglutamine aggregates. Paradoxically, VCP knockdown also diminished polyglutamine aggregate formation. Furthermore, its ATPase activity is required for the re-solubilization and reactivation of heat-denatured proteins, such as luciferase, from insoluble aggregates. We thus propose that VCP functions as a mediator for both aggregate formation and clearance, depending on the concentration of soluble aggregate-prone proteins, indicating that VCP has dual functions as an aggregate formase and an unfoldase. We then examined the potentially elevated aggregate formase activities of mutant VCPs, which have been found to cause IBMPFD (inclusion body myopathy, Paget disease of bone and front-temporal dementia). Indeed, all IBMPFD VCPs showed elevated aggregate formase activities on both polyglutamine and proteasome inhibitor-mediated aggregates. Biochemically, all IBMPFD VCPs showed elevated ATPase activities as well as elevated binding affinities not only for several VCP cofactors, but also for ubiquitinated proteins. Thus controlling the function of VCP, namely decreasing aggregate formase activities and/or increasing unfoldase activities, is expected to be of great benefit for the treatment of IBMPFD and also several neurodegenerative disorders with intracellular protein inclusions.

Background
Nine human inherited neurodegenerative disorders, including HD (Huntington’s disease) and MJD (Machado–Joseph disease), have been shown to be caused by expanded CAG nucleotide repeats, encoding polyglutamine residues in the proteins responsible [1–3]. Expanded polyglutamine tracts, typically of more than 40 repeats, possess an intrinsic ability to aggregate in a polyglutamine length- and concentration-dependent manner [2–4]. The ability of expanded polyglutamines to induce neurodegeneration in mice and cell death in cultured cells appears to be inseparable from their intrinsic ability to aggregate [4–6]. This class of neurodegenerative disorders has thus been collectively called the ‘polyglutamine diseases’. Accordingly, nuclear and/or cytoplasmic expanded polyglutamine-containing aggregates or inclusions have been observed in neurons of patients suffering from essentially all polyglutamine diseases. Accumulation of abnormal proteins has also been observed in various other human neurodegenerative disorders [2] (e.g. prion disease, Alzheimer’s disease, amyotrophic lateral sclerosis and the Lewy body diseases, such as Parkinson’s disease and dementia with Lewy bodies). Thus elucidation of the molecular mechanisms underlying the formation of these abnormal protein aggregates is a major concern for resolution of the pathogenesis, as well as finding novel strategies for curing or preventing these currently non-treatable disorders.

Identification of VCP (valosin-containing protein) as a polyglutamine-interacting protein
During the search for molecules which interact with expanded polyglutamines in mammalian cells, all cultured cells examined were found to contain a protein with a molecular mass of approx. 100 kDa which interacted with the GST (glutathione transferase)-tagged MJD protein containing a 79-residue polyglutamine repeat (GST–MJD79) much more strongly than with GST–MJD containing a 35-residue polyglutamine repeat (GST–MJD35) [7]. This protein was then purified from both COS cells and HeLa...
cells by GST–MJD79-mediated affinity purification. MS and microsequencing analyses revealed that the protein isolated from both cell types was VCP, a member of the AAA (ATPase associated with various cellular activities) class of proteins [7].

In vitro pull-down assays revealed that VCP was no longer able to bind MJD proteins when the polyglutamine repeat portions were deleted [7]. Northern blot analyses of human RNA showed that VCP was ubiquitously expressed in all tissues and throughout the brain [7]. Expression of VCP-(K524A), a dominant-negative mutant, led to cell death with accumulation of ubiquitinated proteins and formation of ER (endoplasmic reticulum)-derived vacuoles with ER stress. Given that these phenotypes have been commonly observed in neurodegeneration, the potential involvement of VCP in neuronal cell death has been suggested [7,8]. It is noteworthy that VCP has also been identified as a genetic modifier of expanded polyglutamine repeat-induced eye degeneration in a Drosophila model of polyglutamine diseases [9,10]. These results strongly suggested the involvement of VCP in the pathogenesis of at least polyglutamine diseases.

Co-localization of VCP with protein aggregates in polyglutamine diseases and also in several other neurodegenerative disorders

Consistent with the in vitro pull-down results, endogenous VCP was found to co-localize with expanded polyglutamine repeat aggregates. An overexpressed FLAG-tagged 79-residue polyglutamine repeat aggregate was found to form aggregates both in the nucleus and in the cytoplasm in neuronally differentiated PC12 cells (pheochromocytoma cells), and to co-localize with endogenous VCP [7]. Immunohistochemical analysis showed that in brain sections from HD and MJD patients, strong VCP signals were detected in the nuclear inclusions [7]. Furthermore, VCP-positive staining of Lewy bodies was also observed in patients suffering from Parkinson’s disease and in patients with dementia with Lewy bodies [7,11], indicating that VCP can recognize a broad range of abnormally folded proteins. VCP co-localization with abnormal protein aggregates observed in other human neurodegenerative disorders was then examined. Indeed, VCP co-localization was observed in the inclusions in motor neurons of SOD1 (superoxide dismutase 1) mutant mice, a mouse model of amyotrophic lateral sclerosis [12], as well as in the neurons of patients suffering from motor neuron disease with dementia [11]. Dystrophic neuritis in Alzheimer’s disease and Marinesco bodies in Parkinson’s disease were also VCP-positive [11]. Consistent with these observations, strong VCP signals were observed in aggresomes, proteasome inhibitor-induced intracellular accumulations of ubiquitinated proteins, of cultured cells [7,13].

VCP in the clearance of abnormal protein aggregates in cultured cells

Given that VCP belongs to the AAA class of ATPases, which includes several proteins functioning in protein quality control, and that VCP itself has been shown to function in such processes, an alternative possibility is that VCP has yet unknown biological effects on these aggregates. We then generated aggresomes or aggregates of expanded polyglutamine repeats, co-expressed GFP (green fluorescent protein)-tagged VCP, and traced the GFP signals after aggregate formation ceased. VCP–GFP co-localized with these protein aggregates, even when expressed after aggregate formation had occurred [13]. The aggregates gradually shrank with continued culture of the cells, but VCP remained co-localized until the aggregates disappeared completely [13]. After this, GFP signals were diffusely distributed throughout the cells [13]. These observations suggest the possibility that VCP is capable of recognizing aggregates, even when aggregates are pre-formed, and remains associated with them until their complete disappearance. The clearance of such aggregates was delayed in cells in which VCP levels were mildly reduced by VCP siRNA (small interfering RNA), or where a dominant-negative VCP mutant was overexpressed, showing that VCP is involved in the processes of clearing the pre-formed aggresomes and expanded polyglutamine repeat aggregates [13].

Chaperone effects of VCP on heat-denatured luciferase

These results suggest the possibility that VCP is able to function as an unfoldase towards aggregates. We then examined whether VCP has chaperone effects on denatured proteins, namely heat-denatured firefly luciferase. Cells expressing luciferase were heat-shocked at 45 °C for 15 min, and then incubated at 37 °C for several hours while new protein synthesis was inhibited by the addition of cycloheximide. Every 1 h, for up to 4 h after the heat shock, cells were harvested and their luciferase activity was measured. In this experiment, we compared the luciferase activity from four types of cells: control HeLa cells, VCP KD (knockdown) HeLa cells, VCP KD HeLa cells with wild-type VCP–GFP re-introduced and VCP KD HeLa cells with VCP(K251A)–GFP re-introduced. VCP KD cells failed to reactivate luciferase after the heat shock, when compared with control cells [13]. However, the re-introduction of VCP–GFP into VCP KD cells led to a significant re-activation of luciferase activity, indistinguishable from that of control cells [13]. To examine whether the ATPase activity of VCP is required for this reactivation, VCP(K251A), an ATPase activity-deficient mutant, was introduced into VCP KD cells. VCP(K251A)–GFP either reduced further or failed to reactivate luciferase activity [13]. These results clearly indicate that VCP is involved in the re-folding and the reactivation of luciferase denatured by heat shock, and that its ATPase activity is essential for this function.

Luciferase levels in the aggregate fraction increased remarkably just after the heat shock in both VCP KD cells and VCP KD cells expressing VCP–GFP, but luciferase levels were found to be reduced in VCP KD cells expressing VCP–GFP than in VCP KD cells 4 h after the heat shock [13]. In VCP KD cells, the amount of luciferase stayed constant in
the pellet fraction. Consistent with this, soluble luciferase levels increased in VCP KD cells expressing VCP–GFP, but not in VCP KD cells [13]. These results indicate that VCP plays an important role in reactivating luciferase through resolubilizing it from aggregates. This is the first clear demonstration that VCP possesses an unfoldase-like activity in vivo.

**VCP in the formation of abnormal protein aggregates in cultured cells**

Surprisingly, VCP behaved in a totally opposite way in aggresome formation, compared with aggresome clearance. In cells treated with VCP siRNA or expressing VCP(K524M), another ATPase activity-deficient VCP, proteasome inhibitors could not efficiently induce aggresome formation [13,14]. Furthermore, VCP siRNA dose-dependently suppressed the formation of aggregates by expanded polyglutamine repeats in PC12 cells. As observed with aggresome formation and clearance, VCP behaved in a totally different way depending on the expanded polyglutamine-expressing phases, namely during or after the expression. VCP was able to enhance aggregate formation during the expression of expanded polyglutamine repeats, and then VCP changed its function to eliminate the aggregates after expression of the expanded polyglutamine repeats was shut down [13]. These results as a whole suggest the possibility that VCP catalyses both aggregate formation and clearance, depending on the concentration of soluble aggregate-prone proteins, rather than on the concentration of already aggregated or insoluble proteins. Namely, during the expression of expanded polyglutamine repeats, it is expected that the concentration of soluble aggregate-prone expanded polyglutamine repeats is high, and after shutting off the expanded polyglutamine repeat expression, the concentration decreases. Indeed, each condition induced aggregate formation and clearance respectively, and both aggregate formation and clearance were inhibited by VCP KD.

**Characterization of IBMPFD (inclusion body myopathy, Paget disease of bone and front-temporal dementia) VCP**

Existence of VCP-positive inclusions has been observed not only in neurons of patients suffering from several neurodegenerative disorders, but also in the muscles of several myopathies with rimmed vacuoles. These findings led to the discovery of the VCP gene as the gene responsible for IBMPFD [15]. To date, nine missense mutations have been identified in the VCP coding region [15–17]. Given that VCP possesses both aggregate-forming and -clearing activities, it is expected that the former activity is dominant over the latter for the VCP mutations involved in IBMPFD. Indeed, cells expressing any of the IBMPFD VCP mutations showed an increased formation of both aggresome and expanded polyglutamine aggregates when compared with cells expressing wild-type VCP. Biochemically, all IBMPFD VCP mutants showed elevated ATPase activities, as well as elevated binding affinities for several VCP cofactors and for ubiquitinated proteins. It is noteworthy that the rimmed vacuoles in muscles contain Aβ (amyloid β-peptide), α-synuclein, tau and ubiquitinated proteins [18–20], which are all found in neuronal inclusions in neurodegenerative disorders. These observations further support and enhance the potential roles of VCP as a common player in the pathogenesis among human neurodegenerative disorders, as well as in human muscular disorders. VCP may also play important roles in several bone disorders.

**Conclusions**

In relation to human disorders, VCP was originally identified as a polyglutamine repeat-interacting protein. Loss of VCP function in cells by the overexpression of dominant-negative VCP or by VCP siRNA treatment led to cell death, suggesting the potential involvement of VCP in neuronal cell death. In the still-surviving neuronal cells, however, VCP has been found to co-localize with several different inclusions in polyglutamine diseases and also in other neurodegenerative disorders, e.g. Parkinson’s disease and amyotrophic lateral sclerosis. We have shown that VCP possesses both aggregate-forming and -clearing activities. Interestingly, in IBMpFD VCP, the aggregate-forming activity appeared to be dominant over the aggregate-clearing activity, in connection with the elevated activities of ATPase and the binding activities to cofactors and ubiquitinated proteins. Thus it is important to find ways to control the function of VCP towards decreasing aggregate formase activities and/or increasing unfoldase activities in vivo. Overcoming this challenge would be a great benefit for the treatment of not only IBMpFD, but also several muscular and neurodegenerative disorders with intracellular protein inclusions.

**References**


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