Valosin-containing Protein (VCP) in Novel Feedback Machinery between Abnormal Protein Accumulation and Transcriptional Suppression*‡

Masaaki Koike‡, Junpei Fukushi‡, Yuzuru Ichinohe‡, Naoki Higashimae‡, Masahiko Fujishiro‡, Chiyomi Sasaki‡, Masahiro Yamaguchi‡, Toshiki Uchihara‡, Saburo Yagishita‡, Hiroshi Ohizumi‡, Seiji Hori‡, and Akira Kakizuka‡

From the ‡Laboratory of Functional Biology, Kyoto University Graduate School of Biostudies & Solution Oriented Research for Science and Technology (JST), Kyoto 606-8501, Japan, the ‡Department of Physiology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan, the ‡Department of Neurology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183-8526, Japan, and the ‡Department of Pathology, Kanagawa Rehabilitation Center, Kanagawa 243-0121, Japan

Abnormal protein accumulation is often observed in human neurodegenerative disorders such as polyglutamine diseases and Parkinson disease. Genetic and biochemical analyses indicate that valosin-containing protein (VCP) is a crucial molecule in the pathogenesis of human neurodegenerative disorders. We report here that VCP was specifically modified in neuronal cells with abnormal protein accumulation; this modification caused the translocation of VCP into the nucleus. Modification-mimic forms of VCP induced transcriptional suppression with deacetylation of core histones, leading to cell atrophy and the decrease of de novo protein synthesis. Preventing VCP nuclear translocation in polyglutamine-expressing neuronal cells and Drosophila eyes mitigated neurite retraction and eye degeneration, respectively, concomitant with the recovery of core histone acetylation. This represents a novel feedback mechanism that regulates abnormal protein levels in the cytoplasm during physiological processes, as well as in pathological conditions such as abnormal protein accumulation in neurodegenerations.

Homeostasis is a fundamental property of organisms and cells that allows them to remain healthy in the face of changes in the environment. Feedback mechanisms are the core machinery for maintaining homeostasis. One example of feedback mechanism is end-product inhibition in enzyme reactions, and another is unfolded protein response (UPR) in ER (1, 2). The goal of UPR is to reduce the amount of accumulated misfolded proteins in the ER (3). Although there have been a number of studies of the elegant feedback mechanisms employed for ER quality control, it is currently unknown whether similar mechanisms exist to reduce the cytoplasmic accumulation of misfolded proteins. Many neurodegenerative diseases (e.g. polyglutamine diseases, AD, PD, and ALS) share pathological features such as accumulation of abnormal proteins, neural cell atrophy, or degeneration, and neuronal cell death (4, 5), suggesting that common molecular mechanisms underlie these disorders. Indeed polyglutamine expansions have been identified in aggregated proteins in nine inherited neurodegenerative disorders, including HD and MJD (also called SCA3), α-synuclein in PD, DLB, and multisystem atrophy, and TDP-43 in certain types of front-temporal dementia, PD without Lewy bodies, and ALS (4–6). These proteins accumulate in the cytoplasm, the nucleus, or both, and have been proposed to trigger multiple cellular responses, such as cell death, proteasomal dysfunction, ER stress, and oxidative stress (7–11), as well as transcriptional dysregulation (12, 13).

Transcriptional regulation of gene expression is often accompanied by histone modifications, such as acetylation, phosphorylation, ubiquitination, and methylation (14, 15). Among these, acetylation of core histones (such as H3 and H4) reflects the open chromatin configuration that allows the transcriptional machinery to easily access target genes and thus promote transcription (14, 16). Several groups have reported that polyglutamine aggregates suppress cellular functions at the transcription level, and that this suppression is accompanied by decreased acetylation of core histones. It has been argued that sequestration of major HATs (e.g. CBP, PCAF, and TAFII130) underlies this transcriptional suppression (12, 13, 17–19). It is notable that decreased acetylation of core histones and transcriptional dysregulation has been observed not only in polyglutamine disease models, but also in PD, ALS, and AD models (20–22). Unlike polyglutamine disease models, sequestration of transcription factors has not been observed in the PD, ALS, and AD disease models, suggesting that an as-yet unknown mechanism may underlie transcriptional dysregulation in these disorders.

Valosin-containing protein (VCP) is a member of the AAA protein family. VCP has been proposed to function in a variety of physiological processes such as cell cycle regulation, mem-
brane fusion, ER-associated degradation (ERAD), and ubiquit-in-mediated protein degradation (23, 24). We previously identified VCP as a binding partner of MJD proteins with expanded polyglutamine tracts (25). Furthermore, VCP has been shown to colocalize not only with polyglutamine aggregates, but also with Lewy bodies in PD and DLB, SOD1 aggregates in ALS, and dystrophic neurites in AD (25–28). Several lines of evidence have shown that VCP can mediate both aggregate formation and clearance, which is reminiscent of yeast Hsp104, another AAA protein (see references in 29). In addition, by genetic screening using Drosophila models of polyglutamine disease, we identified ter94, the Drosophila homolog of VCP, as a genetic modifier of eye degeneration phenotypes induced by expanded polyglutamine tracts (30). VCP has also been shown to be highly modified by S-thiolation, phosphorylation, and acetylation (31–34).

Recently, about a dozen missense mutations in the human VCP gene have been identified as causing inclusion body myopathy with Paget disease of bone and frontotemporal dementia (IBMPFD), an autosomal dominant inherited disease that affects multiple tissues, including muscle, bone, and the cerebral cortex (35–39). Although it is now known that VCP is critically involved in the pathogenesis of several types of human disorders, including neurodegeneration, the detailed molecular mechanisms mediated by VCP in neurodegenerative disorders remain to be elucidated.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal antibodies against VCP were developed in our laboratory and described previously (25, 40). The anti-FLAG antibodies (M2 and M5) were obtained from Sigma; anti-actin antibody from Chemicon; anti-histone H3, H4, acetyl-histone H3 (for acetylated Lys-9 and Lys-14), and acetyl-histone H4 (for acetylated Lys-5) antibodies from Upstate Cell Signaling. Anti-GFP and anti-HA antibodies are from Nacalai and Roche, respectively.

**Cell Cultures and Transfection**—HEK293, NIH3T3, and MCF7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) with 10% fetal bovine serum. PC12 and its derivative cells were maintained on type 4 collagen-coated culture dishes in DMEM (low glucose) with 10% fetal bovine serum. When needed, cells were transfected with expression plasmids using Lipofectamine Plus, Lipofectamine 2000TM, or Cellfectin (Invitrogen). 

**Incorporated radio counts were measured by a liquid scintillation counter.**

**Q64C Transgenic Mice**—The Q79C mice that we had previously created could not be bred due to very severe ataxia (7), but we recently established new polyglutamine transgenic mice, which express an HA-tagged C-terminal portion of MJD protein with 64 glutamine repeats (Q64C) in Purkinje cells by using the L7 gene. In one mouse, the transgene integrated into the X chromosome, and we referred to this mouse as Q64C mouse. Thus, the female transgenic mice showed very mild ataxia, probably due to Lyonization, and were breedable. We could observe two types of Purkinje cells in female cerebellum: cells with atrophic morphologies that expressed Q64C, and cells with normal morphologies, which did not express Q64C.
VCP in Novel Feedback Machinery

(A) 

<table>
<thead>
<tr>
<th></th>
<th>Tak8</th>
<th>TQ15</th>
<th>Tak8</th>
<th>TQ15</th>
</tr>
</thead>
<tbody>
<tr>
<td>butyrate</td>
<td>0 2 4</td>
<td>0 2 4</td>
<td>0 4 0</td>
<td>0 4 0</td>
</tr>
<tr>
<td>FLAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcH3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcH4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Tet-off: day)

(B) 

FLAG-polyQ  
merge  
AcH3  
AcH4

(C) 

![Graph showing AcH3 or AcH4-reduced cells/FLAG-polyQ-positive cells.](image)

(D) 

![Graph showing AcH3 or AcH4-reduced cells/FLAG-polyQ-positive cells.](image)

(E) 

PC12  3T3  293A  MCF7

(F) 

VCP  FLAG-polyQ  phase  merge

(G) 

(H) 

![Graph showing AcH3 or AcH4-reduced cells/FLAG-polyQ-positive cells.](image)
Transgenic Flies—Transgenic flies were created as described previously (30). Genotypes were the followings: GMR-Gal4/+ (A), GMR-Gal4/+; UAS-wtVCP/+ (wtVCP), GMR-Gal4/+; GMR-FlagQ92/+ (Q92), and GMR-Gal4/+; GMR-FlagQ92/UAS-wtVCP (Q92/wtVCP).

Statistical Analysis—Each experiment was conducted at least three times with consistent results. The gel or blot representative of each experiment is presented. The statistical significance was analyzed using Student’s t test.

RESULTS

Polyglutamine Aggregates, VCP Nuclear Translocation, and Histone Deacetylation—We had previously established a PC12 cell line (TQ15 cells) expressing FLAG-tagged 79-glutamate repeats (Q79) under the control of the tet-off promoter (40). Western blot analysis clearly demonstrated that Lys-9 and Lys-14 of H3 and Lys-5 of H4 were deacetylated after the expression of Q79 in a time-dependent manner (Fig. 1A). Addition of butyrate, an inhibitor of histone deacetylases, dramatically recovered or even enhanced the acetylation of histones H3 and H4, which was indistinguishable between cells with or without polyglutamine aggregates (Fig. 1A and supplemental Fig. S1). These observations fit well with the idea that expanded polyglutamine tracts contribute to enhancement of histone deacetylation rather than to diminishment of histone acetylation.

Surprisingly, significantly more cells with cytoplasmic aggregates showed a decrease in H3 and H4 acetylation than cells with nuclear aggregates alone (Fig. 1, B and C). We then transiently expressed NLS- or NES-tagged Q79 in parental PC12 cells and examined the location of aggregates and the acetylation levels of H3 and H4. As expected, all of the aggregates of NLS-Q79 or NES-Q79 were located in the nucleus or in the cytoplasm, respectively (data not shown). Significantly more cells expressing NES-Q79 showed a decrease in H3 and H4 acetylation than cells expressing NLS-Q79 (Fig. 1D and supplemental Fig. S2). This demonstrates that polyglutamine aggregates were able to induce core histone deacetylation, irrespective of whether they were nuclear or cytoplasmic.

We have long examined the relationship between VCP and the accumulation of abnormal proteins such as those containing expanded polyglutamines and noticed that VCP changes its localization from the cytoplasm to the nucleus even in cells with polyglutamine aggregates in the cytoplasm (Fig. 1, E and F), which was most often observed in neuronal cells. We then examined brain sections from our recently established Q64C mice and MJD patients. In Purkinje cells from normal mice, VCP was distributed diffusely throughout the cell. In contrast, VCP was mainly localized within the nucleus in the Q64C-expressing atrophic Purkinje cells of Q64C mice (supplemental Figs. S3 and S4). In the brain sections of MJD patients, nuclear inclusions (NIs) were stained with an anti-1C2 monoclonal antibody (black), and VCP was identified with an anti-VCP polyclonal antibody (brown). We found that nearly 80% of NI-positive neurons showed VCP staining in the nucleus. In contrast, only about 20% of NI-negative neurons showed VCP staining in the nucleus (Fig. 1, G and H). These results showed that there is a correlation between the accumulation of expanded polyglutamines and VCP nuclear localization.

VCP Modification and Atrophic Phenotypes—We have previously shown that VCP is highly modified by phosphorylation and acetylation (34). We thus purified FLAG-VCP from HEK293T cells expressing expanded polyglutamine tracts, analyzed it by LC/MS/MS, and found that there were several amino acids, Ser-612, Thr-613, and Lys-614, were modified simultaneously by phosphorylation, phosphorlylation, and acetylation, respectively (Fig. 2). These sequential modifications were not observed in VCP purified from cells expressing normal lengths of polyglutamines. The same sequential modifications were observed, although less frequently, in purified FLAG-VCP from MG132-treated cells (34). In the absence of MG132 treatment, these sequential modifications were not detected by LC/MS/MS analysis.

To elucidate the biological significance of these VCP modifications, we introduced amino acid substitutions mimicking these modifications into VCP. Ser-612, Thr-613, and Lys-614 were substituted with aspartic acid (D) (a negatively charged amino acid), glutamic acid (E) (another negatively charged amino acid), and glutamine (Q) (an uncharged amino acid), respectively, in various combinations. The resulting substituents were referred to as: VCP(DTK), VCP(SEK), and VCP(STQ) (single substitutions); VCP(DEK) (double substitution); and VCP(DEQ) (triple substitution). In addition, alanine was introduced as several control substitutions. The resulting substituents were referred to as: VCP(AAK), VCP(AAQ), VCP(DEA), and VCP(AAA). These substituents, as well as wild-type VCP (wtVCP), were expressed as GFP-tagged proteins in PC12 cells. We were thus able to examine these exogenously expressed VCPs with live cell-imaging analysis as well as confocal imaging analysis. wtVCP was mainly localized in the cytoplasm. However, several modification-mimic VCPs
localized in the nucleus with different degrees. VCP(DEQ) was most often localized within the nucleus, followed by VCP(DEA) and VCP(DEK) (Fig. 3, A and B). VCP (AAA), VCP(AAQ), and VCP(AAK) were mostly excluded from the nucleus; less than 5% of transfected cells showed nuclear localization of these substituents (Fig. 3, A and B).

Surprisingly, 5 days after transfection, ~30% of the PC12 cells expressing VCP(DEQ) showed shrinkage of cell volume with neurite retraction (Fig. 3, A and C). We called this an “atrophic phenotype”. The atrophic phenotype was most prominent in cells expressing VCP(DEQ), followed by VCP(DEA) and VCP(DEK) (Fig. 3C), and it was exactly correlated with
FIGURE 3. Phenotypes of PC12 cells induced by modification-mimic forms of VCP. A, immunocytochemical analysis of PC12 cells expressing GFP-tagged wild-type VCP, VCP(DEQ), and VCP(AAA). VCP(DEQ), but not wild-type VCP or VCP(AAA), localized within the nucleus. Four days after transfection, PC12 cells were fixed and stained with anti-GFP (green), anti-β-tubulin (red), and TOTO-3 (blue). PC12 cells expressing VCP(DEQ) showed retracted neurites and were small in size (atrophic phenotype). Bars, 20 μm. B, quantification of GFP-tagged wild-type and various forms of VCP localization in PC12 cells. At least 200 GFP-positive cells were imaged randomly and scored for nuclear localization of GFP signals. C, quantification of atrophic phenotypes in PC12 cells expressing GFP-tagged wild-type and various forms of VCP. At least 200 GFP-positive cells were imaged randomly and scored for atrophic phenotypes. D, quantification of atrophic phenotypes in PC12 cells expressing GFP-tagged wild-type or various forms of VCP, some of which were also tagged with NLS or NES. At least 200 GFP-positive cells were imaged randomly and scored for atrophic phenotypes. E, quantification of immunocytochemical analysis for decreased histone acetylation in cells expressing GFP-tagged wild-type or various forms of VCP. At least 100 GFP-positive cells were imaged randomly and scored for decreased histone acetylation.
their nuclear translocation (Fig. 3, B and C). More cells expressing VCP(DEQ) showed an atrophic phenotype with increasing time, but remained alive even 7 days after transfection (data not shown). We could observe very little of the atrophic phenotype in cells expressing wtVCP (less than 5%).

We next sought to determine the cause of the atrophic phenotype, whether it was VCP nuclear localization, VCP amino acid substitution, or both. To this end, we expressed NLS- and NES-tagged wtVCP and several substituents in PC12 cells, and examined the phenotypes. All of the NLS-tagged VCPs translocated to the nucleus, and all of the NES-tagged VCPs stayed in the cytoplasm (data not shown). NLS-VCP(DEQ) induced the atrophic phenotype most frequently (more than 50% of cells) (Fig. 3D). The expression of other NLS-tagged VCPs (wtVCP, VCP(AAK), and VCP(AAA)) either did not result in or only marginally increased the atrophic phenotype (Fig. 3D). Conversely, NES-VCP(DEQ) did not result in the atrophic phenotype (Fig. 3D). These data demonstrate that both the modifications and the nuclear localization of VCP were necessary for the atrophic phenotype. Taken together, the above results suggested that modified VCP mediates a signal from the cytoplasm to the nucleus, leading to histone deacetylation. Indeed, deacetylations of H3 and H4 histones were observed in cells expressing VCP(DEQ) and NLS-VCP(DEQ), but not or only marginally in cells expressing wtVCP or VCP(AAA), even in the presence of NLS (Fig. 3E and supplemental Fig. S5).

**VCP Modification and the Suppression of Transcription and Protein Synthesis**—We next examined whether VCP(DEQ) expression could suppress steady-state mRNA levels in a transient transfection assay. For this experiment, we prepared an expression vector for luciferase RNA with the rat or human ribosomal RNA (rRNA) promoter (pRrD-Luc or pHrD-Luc, respectively), as an internal transfection control. Northern blot analysis revealed that luciferase RNA levels from both of the rRNA promoters were not affected by the addition of actinomycin D (an inhibitor of RNA polymerase I) or butyrate, or by wtVCP or VCP(AAA) expression. However, the addition of actinomycin D (an inhibitor of RNA polymerase I) completely
blocked the luciferase RNA expression (Fig. 4, A and B, and supplemental Fig. S6).

We then investigated whether VCP(DEQ) affected the promoter activities of different genes transcribed by RNA polymerase II. HEK293T cells were transfected with expression vectors for GFP, wtVCP-GFP, or VCP(DEQ)-GFP, along with pHRD-Luc and a well-used vector that expresses luciferase 2. We examined levels of luciferase 2 mRNA by Northern blot and found that luciferase 2 mRNA from all of the tested promoters (e.g. those from the cytomegalovirus (CMV) and EF-1α genes) was suppressed by the expression of VCP(DEQ)-GFP, but not wtVCP-GFP (Fig. 4A, and not shown). Interestingly, this suppression was not fully ameliorated by the addition of butyrate (Fig. 4A). Similar decreases in mRNA levels were observed in cells transfected with the Q79 expression vector, but not the Q35 expression vector or empty vector (Fig. 4B). Again, this suppression was not fully ameliorated by the addition of butyrate (Fig. 4B).
VCP in Novel Feedback Machinery

A

VINQLTEMGMSTKK  Homo sapiens
VINQLTEMGMSTKK  Rattus norvegicus
VINQLTEMGMSTKK  Mus musculus
VINQLTEMGSMIKK  Xenopus laevis
VINQLTEMGMGAKK  Drosophila melanogaster (ter94)
VINQVLMGEMNAKK  Caenorhabditis elegans (Cdc48.1)
VLNQITEMGMNAKK  Arabidopsis thaliana (Cdc48p)
V-NQLTEMGMNAKK  Saccharomyces cerevisiae (Cdc48p)

B

mock

VCP

NES-VCP

NLS-VCP

C

D

VCP-GFP  AcH3  HA-polyQ  merge

VCP

NES-VCP

NLS-VCP

E

AcH3-reduced cells / GFP-positive cells (%)

GFP  WT  NES-WT  NLS-WT

polyQ (-)  polyQ (+)
We then performed a similar set of experiments using Tak8 (parental PC12 cells), Tv10 (PC12 cells expressing GFP-tagged wtVCP under the control of the tet-off promoter), DEQ12 (PC12 cells expressing GFP-tagged VCP(DEQ) under the control of the tet-off promoter), and AAA26 cells (PC12 cells expressing GFP-tagged VCP(AAA) under the control of the tet-off promoter) (supplemental Fig. S7). Consistent with the results from the HEK293T cells, luciferase 2 mRNA levels from the CMV promoter were suppressed in DEQ12 cells but not in Tak8, Tv10, or AAA26 cells 3 days after the removal of tetracycline. Again, this suppression was not fully ameliorated by the addition of butyrate (Fig. 4C).

The above results indicate that expanded polyglutamine expression induced VCP modifications, which in turn induced suppression of transcription from promoters transcribed by RNA polymerase II but not RNA polymerase I. Thus, we expected de novo protein synthesis to be reduced. To investigate this possibility, we examined the levels of de novo protein synthesis in Tak8, Tv10, DEQ12, and AAA26 cells by measuring [35S]methionine/cysteine incorporation into newly synthesized proteins. Indeed, we confirmed that de novo protein synthesis was significantly decreased in DEQ12 cells, but not in Tv10 or AAA26 cells 5 and 6 days after removal of tetracycline (Fig. 4D).

**Prevention of Polyglutamine-induced Phenotypes by NES-VCPs in PC12 Cells**—We next examined the potential dominant-negative effects of VCP(AAA) on endogenous VCP. In Tak8 cells (parental PC12 cells), tetracycline removal did not affect the intracellular distribution of GFP-tagged wtVCP, VCP(AAA), NES-wtVCP, or NES-VCP(AAA) (Fig. 5A). Approximately 20% of cells with GFP signals in the nucleus were expressing wtVCP or VCP(AAA); very few cells with nuclear GFP were expressing NES-wtVCP or NES-VCP(AAA). Similar expression patterns were observed in TQ15 cells (PC12 cells expressing FLAG-Q79 under the control of the tet-off promoter) (40) in the presence of tetracycline. However, tetracycline removal from TQ15 cells, resulting in expression of Q79, induced nuclear translocation of both wtVCP (~80% of cells) and VCP(AAA) (~70% of cells) (Fig. 5A). In contrast, only marginal nuclear translocation of NES-wtVCP or NES-VCP(AAA) was observed in TQ15 cells 4 days after removal of tetracycline (Fig. 5A). Consistent with these results, overexpression of NES-wtVCP or NES-VCP(AAA) was able to suppress the expanded polyglutamine-induced deacetylation of histone H3 (Fig. 5, B and C, and not shown), as well as neurite retraction (Fig. 5D).

**Prevention of Polyglutamine-induced Phenotypes by wtVCP in Drosophila**—It is notable that Ser-612 and Thr-613 of mammalian VCP could not be phosphorylated in Drosophila, it was interesting to see whether mammalian VCP was also able to translocate into the nucleus in Drosophila cells in the presence of polyglutamine aggregates. We thus examined this possibility using S2R+ cells (41), a Drosophila cell line, by expressing FLAG-ter94 with HA-tagged Q92 (Q92). In the absence of Q92 expression, ter94 resided mainly in the cytoplasm. In the presence of Q92 aggregates, ter94 translocated into the nucleus (Fig. 6B, uppermost panels). However, wtVCP as well as NES-wtVCP stayed in the cytoplasm, even with co-expression of ter94 in cells with Q92 aggregates (Fig. 6, B and C). More importantly, not only NES-wtVCP but also wtVCP significantly prevented ter94 nuclear translocation in cells with Q92 aggregates (Fig. 6, B and C). Consistent with this, in cells expressing wtVCP or NES-wtVCP, acetylation levels of core histones were also significantly recovered even with Q92 aggregates (Fig. 6, D and E). It is noteworthy that in Drosophila S2R+ cells, NLS-VCP itself could induce deacetylation of core histones with its nuclear localization (Fig. 6, D and E), although this was only marginally observed in mammalian cells (Fig. 3E).

We previously showed that overexpression of ter94, Drosophila VCP, enhanced polyglutamine-induced eye degenerations (30). It was evident, in contrast, that overexpression of mammalian VCP (wtVCP) in fly eyes clearly suppressed eye degenerations when co-expressed with expanded polyglutamines, without apparently affecting the levels of polyglutamine aggregates (Fig. 7, A and B). In degenerated eyes, endogenous ter94 located in the nucleus (Fig. 7C). In cured eyes, however, overexpressed wtVCP and ter94 predominantly resided in the cytoplasm (Fig. 7C), and acetylation levels of core histones were significantly recovered (Fig. 7, D and E). These results, altogether, indicated that in Drosophila cells wtVCP could function in a way similar to NES-wtVCP in mammalian cells, and thus overexpression of wtVCP could mitigate polyglutamine-induced eye degeneration in Drosophila. Furthermore expression of VCP(DEQ) itself in fly eyes induced late onset eye degenerations (supplemental Fig. S8). In degenerated eyes, clear reductions in acetylation levels of core histones were observed (supplemental Fig. S8).

**DISCUSSION**

Several reports have shown that expanded polyglutamine expression decreases the acetylation of core histones such as H3 and H4 (12, 13, 17, 19). We first confirmed this observation in our cell culture model of polyglutamine disease (40). Indeed, the experiments presented here using anti-acetylated H3 (for acetylated Lys-9 and Lys-14) and H4 (for acetylated Lys-5) anti-

---

**FIGURE 6. Prevention of polyglutamine-induced ter94 nuclear translocation and histone deacetylation by wtVCP in Drosophila S2R+ cells. A**, amino acid alignment of modified residues in VCP among different species. Lys-614 was widely conserved among different species; Ser-612 and Thr-613 were conserved only in mammals. B, immunocytochemical analysis of S2R+ cells transfected with FLAG-ter94 and HA-Q79 (HA-polyQ) with or without GFP-tagged wtVCP/VP, NES-wtVCP(NES-VCP), and NLS-wtVCP(NLS-VCP). FLAG-ter94 was detected with an anti-FLAG M5 antibody, Q79 with an anti-HA antibody, VCPs with an anti-GFP antibody. Merged images are also shown in the right panels with indicated colors. White arrowheads show cells with Q79 aggregates. Bars, 10 μm. C, quantification of immunocytochemical analysis in B. At least 200 FLAIR-positive cells were imaged randomly and scored. **, p < 0.01; n.s., not significant. D, immunocytochemical analysis of S2R+ cells transfected with HA-Q79 (HA-polyQ) with GFP-tagged wtVCP (VCP), NES-wtVCP (NES-VCP), and NLS-wtVCP (NLS-VCP). Q79 was detected with an anti-HA antibody, acetyl-H3 (AchH3) with an anti-acetylated histone H3 antibody, VCPs with an anti-GFP antibody. Merged images are also shown in the right panels with indicated colors. White arrowheads indicate cells with Q79 aggregates. Bars, 10 μm. E, quantification of immunocytochemical analysis in D. At least 100 GFP-positive cells were imaged randomly and scored. **, p < 0.01; *, p < 0.05; n.s., not significant.
bodies clearly demonstrate a Q79-dependent decrease in H3 and H4 acetylation. In our mouse model of polyglutamine disease (Q64 mice), clear decreases in H4 acetylation were also observed. Addition of butyrate, an inhibitor of HDAC, dramatically recovered or even enhanced the acetylated levels of histones H3 and H4 in PC12 cells, so that cells with or without expanded polyglutamine expression indistinguishable. These observations fit well with the idea that expanded polyglutamine tracts contribute to enhancement of histone deacetylation rather than to diminishment of histone acetylation.

Sequestration of major transcription factors such as CBP within polyglutamine aggregates has been proposed to be the main mechanism underlying transcriptional suppression in aggregate-containing cells (17, 44). Because these transcription factors, either by themselves or by physical interactions, contribute to HAT activation, this putative mechanism would lead to a diminishment of histone acetylation rather than to an enhancement of histone deacetylation, and was in direct contrast with our observations. Furthermore, irrespective of the intracellular localization of polyglutamine aggregates, we observed clear decreases in the acetylated levels of histones H3 and H4. More surprisingly, NES-tagged Q79 could induce stronger histone deacetylation than could NLS-tagged Q79. These observations strongly suggest the existence of a mechanism other than sequestration of transcription factors, in which expanded polyglutamine expression, even in the cytoplasm, leads to H3 and H4 deacetylation, probably via HDAC activation.

In cells expressing expanded polyglutamines, three sequential amino acids in VCP, Ser-612, Thr-613, and Lys-614, were modified by phosphorylation, phosphorylation, and acetylation, respectively. We then examined the biological significance of these modifications by creating and expressing several modification-mimic forms of VCPs, such as VCP(DEQ) and VCP(DEK), in PC12 cells. We also created several control substitutions with alanine, e.g. VCP(DEA) and VCP (AAA). Although these substitutions may not perfectly represent modified or non-modified protein configurations, this method has been well-used in elucidating biological functions of the modified or non-modified proteins, respectively, such as kinases, kinase substrates, tubulin, histones etc (45–47).

We found that in more than 95% of cells, exogenously expressed wtVCP stayed in the cytoplasm. In contrast, in more than 60% of transfected cells, exogenously expressed VCP-DEQ was translocated to the nucleus, followed by VCP-DEA (50%) and VCP-DEK (25%). In accordance with this, FLAG-tagged VCP(DEQ), but not wtVCP, could co-precipitate endogenous importins (data not shown). These results strongly suggest that sequential modifications of Ser-612, Thr-613, and Lys-614 were responsible for the nuclear translocation of VCP. Unexpectedly, these modifications also induced neurite retraction and shrinkage in PC12 cells (~30% of cells), which was also observed in PC12 cells expressing expanded polyglutamine tracts. This atrophic phenotype was not induced by forced expression of NLS-tagged wtVCP in the nucleus or by forced expression of NES-tagged VCP(DEQ) in the cytoplasm. In agreement with these results, forced expression of NLS-tagged VCP(DEQ) in the nucleus further increased the atrophic phenotype in cells (~60% of cells).

The above characteristics of VCP suggest that it might be a mediator of histone deacetylation in response to expanded polyglutamines. Indeed, expression of VCP(DEQ), but not wtVCP or VCP(AAA), induced deacetylation of H3 and H4. More importantly, in mammalian cells NES-wtVCP and NES-VCP(AAA), but not VCP(AAA), inhibited the nuclear translocation of endogenous VCP in cells expressing expanded polyglutamines, and thus could block expanded polyglutamine-induced deacetylation of H3 and H4, as well as the atrophic phenotype. In Drosophila, however, wtVCP but not ter94 could prevent ter94 nuclear translocation in a way similar to NES-wtVCP or NES-VCP(AAA) in mammalian cells. Since VCP forms hexameric structure, the observed three sequential modifications of VCP in mammalian cells would not occur so frequently. However, once such modifications occur even in a single or double protomers, the VCP hexamer may be transported into the nucleus. Thus, VCP(AAA) could not suppress the nuclear translocation of the VCP hexamer. In Drosophila cells, however, the presence of the unmodified mammalian VCP in the ter94 hexamer did prevent the nuclear translocation of the hexamer even in the presence of polyglutamine aggregates. This might be due to the creation of certain conformations unfavorable for the nuclear translocation of the hexamer by the incorporation of unmodified mammalian VCP. This idea remains to be clarified. In contrast, expression of VCP(DEQ) itself in fly eyes induced eye degenerations, with decreased core histone acetylations. These results demonstrate that VCP is indeed a major mediator of histone deacetylation, as well as a major molecule causative for the atrophic phenotype or eye degenerations in cells expressing expanded polyglutamines. In our mouse model of polyglutamine disease (Q64 mouse), Purkinje cells with nuclear polyglutamine aggregates clearly showed nuclear VCP localization and decrease of acetylation of histone H4. Recently, Reina et al. demonstrated that MJD protein/ataxin 3 translocated into the nucleus immediately after the heat shock. In this condition, VCP appeared to stay in the cytoplasm (48). It would also be interesting to see whether histone deacetylation could be observed after the heat shock.

Until now, at least 14 different VCP mutants have been reported to cause IBM/PED. The mutated amino acids include R93C, R95C, R95G, R155C, R155H, R155P, G157R, R159H, R159C, R191Q, L198W, A232E, T262A, and N387H (35–39).
VCP in Novel Feedback Machinery

Except for Thr-262, all other mutations were not observed at serine, threonine, tyrosine, or lysine. Since alanine substitution cannot mimic phosphorylation, all these mutations are not able to be mimicked by post-translational modifications, such as phosphorylations or acetylations. It is notable that all mutations relatively reside at N-terminal portion of VCP. Indeed, these mutant VCPs not only showed enhanced binding abilities to VCP cofactors (e.g. Ufd1 and Npl4) and ubiquitinated proteins, but also showed enhanced aggregate-forming activities (Refs. 28, 49).3 Regarding with these characteristics, we could not observe clear difference between wild-type VCP and VCP(DEQ).4 Thus, VCP(DEQ) did not appear to be involved in aggregate formation more positively than wild-type VCP or IBMPFD-causing VCPS.

Treatment of cells expressing expanded polyglutamine or VCP(DEQ) by butyrate did result in a recovery from the H3 and H4 deacetylated state to highly acetylated states. However, this treatment only marginally increased transcription from the CMV promoter and several other promoters, such as the EF-1α promoter. These results clearly indicate that the observed transcriptional suppression was mostly mediated by as-yet unknown HDAC inhibitor-insensitive mechanisms.

Based on the results of this study, we propose a novel feedback mechanism linking the accumulation of cytoplasmic misfolded proteins and transcriptional suppression, in which VCP performs an essential role. Intracellular accumulation of abnormal proteins such as expanded polyglutamines induces VCP modification at Ser-612, Thr-613, and Lys-614, which allows VCP to translocate to the nucleus. Following nuclear VCP translocation, general transcription is suppressed by as-yet unknown mechanisms, resulting in the inhibition of de novo protein synthesis. This inhibition leads to a decrease in the production of new misfolded proteins, and thus allows to cells to dissolve or degrade the accumulated misfolded proteins by cellular mechanisms such as chaperones, proteasomes, and autophagies. This mechanism would work well when misfolded protein accumulation is transient. However, when misfolded proteins are continuously provided, as in the case of neurodegenerative disorders, inhibition of de novo protein synthesis continues for many years or even decades, leading to a gradual decrease in cell mass or shrinkage of affected neurons. Indeed, overexpression of VCP(DEQ) in fly eyes could induce late-onset rough-eye phenotypes. This VCP-mediated feedback mechanism may be a common mechanism underlying neurodegenerative disorders in which there is an intracellular accumulation of abnormal proteins such as polyglutamines, α-synuclein etc.

Acknowledgments—We thank K. Kuroiwa for technical assistance and our laboratory members for valuable discussions. We also thank Drs. T. Kanda and S. Mori (Japan National Institute of Infectious Disease) for pHrD-Luc.

REFERENCES

32. Livingstone, M., Ruan, H., Weiner, J., Clauser, K. R., Strack, P., Jin, S.,