ATPase Activity of p97/Valosin-containing Protein Is Regulated by Oxidative Modification of the Evolutionally Conserved Cysteine 522 Residue in Walker A Motif

Masakatsu Noguchi1‡, Takahiro Takata1‡, Yoko Kimura3, Atsushi Manno1‡, Katsuhiro Murakami1‡, Masaki Koike1‡, Hiroshi Ohizumi1, Seiji Hori3, and Akira Kakizuka1,2‡

From the 1Laboratory of Functional Biology, Kyoto University Graduate School of Biostudies and Solution Oriented Research for Science and Technology (JST), Kyoto 606-8501, Japan and the 2Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan

Valosin-containing protein (p97/VCP) has been proposed as playing crucial roles in a variety of physiological and pathological processes such as cancer and neurodegeneration. We previously showed that VCP(K524A), an ATPase activity-negative VCP mutant, induced vacuolization, accumulation of ubiquitinated proteins, and cell death, phenotypes commonly observed in neurodegenerative disorders. However, any regulatory mechanism of its ATPase activity has not yet been clarified. Here, we show that oxidative stress readily inactivates VCP ATPase activity. With liquid chromatography/tandem mass spectrometry, we found that at least three cysteine residues were modified by oxidative stress. Of them, the 522nd cysteine (Cys-522) was identified as the site responsible for the oxidative inactivation of VCP. VCP(C522T), a single-amino acid substitution mutant from cysteine to threonine, conferred almost complete resistance to the oxidative inactivation. In response to oxidative stress, VCP strengthened the interaction with Npl4 and Ufd1, both of which are essential in endoplasmic reticulum-associated protein degradation. Cys-522 is located in the second ATP binding motif and is highly conserved in multicellular but not unicellular organisms. Cdc48p (yeast VCP) has threonine in the corresponding amino acid, and it showed resistance to the oxidative inactivation in vitro. Furthermore, a yeast mutant (cdc48Δ) was shown to be susceptible to oxidants-induced growth inhibition and cell death. These results clearly demonstrate that VCP ATPase activity is regulated by the oxidative modification of the Cys-522 residue. This regulatory mechanism may play a key role in the conversion of oxidative stress to endoplasmic reticulum stress response in multicellular organisms and also in the pathological process of various neurodegenerative disorders.

Various human neurodegenerative disorders, such as polyglutamine diseases, Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis, have distinct clinical symptoms, but they share several pathological features such as accumulation of abnormal proteins or deposits of ubiquitinated proteins, formation of cytoplasmic vacuoles, and neuronal cell degeneration/death if not observed together in all disorders (1, 2). These observations suggest a potential link between neuronal degeneration/death and dysfunction of the protein quality control system (3) and/or the protein degradation pathway via the ubiquitin-proteasome system (2, 4). Consistently, several inherited neurodegenerative disorders have been shown to be caused by mutations in genes that regulate the ubiquitin-proteasome system (5–7).

We previously identified VCP2 as a binding partner of the MJD protein with expanded polyglutamines (8, 9), which causes Machado-Joseph disease (10), the most common inherited spinocerebellar ataxia (2). VCP, a member of the AAA family proteins, is one of the most abundant intracellular proteins, with a molecular mass of 97 kDa and consists of the N-terminal (N) domain and two ATPase domains (D1 and D2) (11). VCP has been proposed to function in a variety of physiological processes such as the cell cycle, membrane fusion, and ubiquitin-proteasome proteolysis, including ERAD (12–17). Immunohistochemical examinations demonstrated that VCP co-localized with abnormal protein aggregates or ubiquitin-positive inclusions observed in several human neurodegenerative disorders, such as nuclear inclusions in Huntington disease (9), Lewy and Lewy bodies in Parkinson disease (18), intracellular inclusions in motor neuron disease and dementia (18), dystrophic neurites of the senile plaque in Alzheimer disease (18), etc. These results have led us to propose that VCP functions as a sensor for the accumulation of misfolded proteins in cells (2, 17).

Concomitantly, by genetic screening using our Drosophila models of the human polyglutamine disease, we identified ter94, Drosophila VCP, as a modifier of eye degeneration phenotypes induced by expanded polyglutamines (19). Moreover, VCP(K524A), an ATPase activity-negative mutant has been shown to cause ER stress, vacuole formation, and accumulation of ubiquitinated proteins in the membrane fraction followed by cell death (17). These phenotypes are very similar to those observed in the pathology of several human neurodegenerative disorders (see above); we have thus called VCP vacuole-creating protein (9, 18). Consistent with these phenotypes, VCP ATPase activities have been shown to be essential in ERAD (15–17). It has been suggested that excessive accumulation of misfolded proteins inactivates VCP ATPase activity, leading to the pathological processes of neuronal cell death in...
these neurodegenerative disorders (2, 17). Therefore, it is important to clarify the regulatory mechanism of VCP ATPase activity. However, no molecular mechanism in the regulation of VCP ATPase activity has as yet been demonstrated. VCP has also been proposed to be involved in other pathological conditions such as advanced cancers (20, 21) and inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (22).

Many lines of evidence have suggested crucial involvement of oxidative stress in a variety of physiological processes as well as pathological processes such as aging, cancer, diabetes, and several human neurodegenerative disorders (23). Especially in Parkinson disease, oxidized proteins are observed in Lewy bodies from early stages (24). Drugs such as 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, paraquat, rotenone, etc., which can specifically kill the dopamine neurons in the substantia nigra and, thus, are used in the creation of Parkinson disease models, have been shown to create oxidative stress; antioxidants can inhibit cell death caused by these drugs (24, 25). Indeed, several drugs that are known to function as antioxidants have certain, if not strong efficacy on several neurodegenerative disorders (26, 27). However, no target molecule of oxidative stress has as yet been identified in such pathological processes.

Recent studies have revealed that oxidative stress, especially NO, induces cell death via ER stress (28, 29). However, the biological significance and molecular mechanisms that link oxidative stress to ER stress response have not yet been clarified. Recently, it has been shown that several enzymatic activities are regulated by oxidative modifications of cysteine residues, e.g. those in caspase-3, OxyR, and protein-tyrosine phosphatase 1B (30–32). These modifications are collectively called S-thiolation (31), a reversible post-translational modification of cysteine residues that includes disulfide bond formation, S-nitrosylation, S-glutathionylation, and S-hydroxylation. The accumulation of S-thiolated proteins has been observed in response to oxidative stress in vivo (33, 34).

In this paper we report that VCP was modulated and regulated by oxidative stress. VCP ATPase activity was inhibited by various oxidants. With LC/MS we found several cysteine residues that were modified under such conditions. Among them, we identified the Cys-522 as the modification site responsible for the oxidative inactivation. This study not only clarifies the regulatory mechanism of VCP ATPase activity for the first time but also provides the novel idea that oxidative modulation of VCP is a crucial event that links oxidative stress and ER stress response in certain physiological as well as pathological conditions such as neurodegeneration.

**MATERIALS AND METHODS**

**Expression and Purification of His-VCP**—cDNA encoding His-VCP or His-Cdc48p was subcloned in a baculovirus expression vector or pDEST26 (N-His tag), a mammalian expression vector (Invitrogen). Recombinant His-VCPs and His-Cdc48p were expressed in insect SF-9 cells or HEK293T cells by transfection and were purified via subsequent procedures (17); transfected cells were lysed in a Nonidet P-40 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(aminomethylene)-benzenesulfonfonyl fluoride, 20 mM benzamidine, 40 μM phenylmethanesulfonyl fluoride, 0.5 mM NaF, 0.5 mM NaVO4, 0.5 mM NaPPi, and a protease inhibitor mixture (Nacalai Tesque); the lysates were loaded onto nickel-chelated HiTrap chelating columns (Amersham Biosciences), and the columns were washed with a buffer containing 500 mM NaCl, 50 mM potassium phosphate (pH 7.8), and 50 mM imidazole. After the recombinant VCPs were eluted with a 50–500 mM imidazole gradient, VCPs were kept in a storage buffer (50 mM Tris–HCl (pH 8.0) and 20% glycerol).

**Antibodies**—Anti-VCP, anti-p47, anti-Npl4, and anti-Ufd1 antibodies were developed by the standard procedures described previously (9, 17). An anti-Cdc48p antibody was raised against a Cdc48p peptide of HPDQYTKFLGPSK. A rat polyclonal anti-GSH antibody (Chemicon), mouse monoclonal anti-actin and anti-polyubiquitin antibodies (Chemicon), and a rabbit polyclonal anti-CHOP antibody (Santa Cruz Biotechnology) were purchased.

**Measurement of the ATPase Activities**—The ATPase activities of VCP were measured in 20 μl of the ATPase assay buffer (20 mM HEPES (pH 7.4), 50 mM KCl, 5 mM MgCl2) with 100 μM γ-[32P]ATP (0.5 Ci/μmol)(Amersham Bioscienes) at 37 °C for 10 min following the procedure already described (35). After incubation, the reaction was quenched by the addition of 200 μl of 7% ice-cold trichloroacetic acid solution with 1 mM K2HPO4 and then 50 μl of solution A (3.75% ammonium molybdate, 0.02 M silicotungstic acid in 3 N H2SO4) and 300 μl of n-butyl acetate were added to the reaction. The samples were mixed and then centrifuged at 12,000 × g for 1 min. Then, 200-μl aliquots from the upper organic phases were taken, and their radioactivity was determined with a liquid scintillation counter for β-radiation, which determined the amounts of [32P] released.

**Peptide Mass Finger Printing Method**—After DTT treatment, recombinant VCP was incubated with oxidants and then treated with 10% trichloroacetic acid on ice for 20 min, washed with acetone, and dried. Next, the samples were treated with 55 mM IAA in ST buffer (1% SDS, 50 mM Tris–HCl (pH 7.5)) for 15 min, dialyzed in 20 mM NH4HCO3 and treated with 12.5 μg/ml trypsin 37 °C for 3 h. The tryptic-digested peptides were analyzed by LC/MS (Waters, 2795 separation module/Thermo Finningan, LCQ Deca XP plus).

**Purification of FLAG-VCP**—HEK293T cells were transfected with FLAG-VCP expression vectors. Forty-eight hours after transfection cells were washed with ice-cold phosphate-buffered saline with 1.5 mM CaCl2 and 0.5 mM MgCl2 and then lysed in a Triton lysis buffer (25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 1% Triton X-100) containing 40 μM phenylmethanesulfonyl fluoride, 0.5 mM NaF, 0.5 mM NaVO4, 0.5 mM NaPPi, and a protease inhibitor mixture (Nacalai Tesque). After removing the debris by centrifugation for 30 min at 12,000 × g, the supernatants were mixed with anti-FLAG M2 affinity gel (Sigma) and then stirred at 4 °C overnight. Then the gels were rinsed with Tris-buffered saline (25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl2), and FLAG-VCP was eluted with the 40 μg of the FLAG peptide (Sigma).

**Detection of S-Glutathionylation of Recombinant His-VCP**—Purified His-VCP was incubated with 100 μM GSH, 500 μM diamide plus 100 μM GSH, 10 mM GSSG, 10 mM GSNO at 37 °C for 30 min. Alternatively, His-VCP was incubated with 98 μM GSH plus 2 μM [35S]GSH (30 Ci/μmol)(Amersham Biosciences) with and without 500 μM diamide at 37 °C for 30 min. With or without subsequent treatment with 30 mM DTT, the samples were mixed with SDS-PAGE sample buffer and then subjected to non-reducing 7.5% SDS-PAGE (300 ng of VCP/lane), and VCP was detected by Western blot analysis with an anti-GSH antibody (Chemicon) or by autoradiography.

**Labeling of S-Glutathionylated Proteins with [35S]Cysteine in HEK293T Cells**—HEK293T cells were transfected with FLAG-VCP expression vectors. Twenty-four hours after transfection, culture medium was changed to Dulbecco’s modified Eagle’s medium lacking sulfur-containing amino acids supplemented with 10% dialyzed serum at 37 °C for 16 h; they were then further incubated in the presence of cycloheximide (50 μg/ml) for additional 1 h at 37 °C and labeled with 30
Red-ox Regulation of VCP ATPase Activity

FIGURE 1. Oxidative stress induced modification and inactivation of VCP. A, diamide plus GSH, GSSG, or GSNO induced S-glutathionylation of VCP in vitro. Recombinant VCPs were treated with GSH, diamide, diamide plus GSH, GSSG, or GSNO. After treatment the samples were subjected to non-reducing 7.5% SDS-PAGE, and VCPs were detected by Western blot analysis with anti-VCP and anti-GSH antibodies. The observed S-glutathionylation were removed by subsequent DTT treatment. B-S, S-glutathionylation of VCP was detected by autoradiography. VCP and bovine serum albumin (BSA) as a positive control were incubated with [35S]cysteine with and without diamide and subjected to non-reducing 7.5% SDS-PAGE followed by autoradiography (Autoradiography and Coomassie Brilliant Blue staining (CBB stain). Bovine serum albumin is reportedly modified by 5-glutathionylation (67). C, diamide induced VCP S-glutathionylation in HEK293T cells. FLAG-VCP expressing HEK293T cells were metabolically labeled with [35S]cysteine. In the presence of cycloheximide most [35S]cysteines were used in GSH synthesis (38).

After diamide treatment, FLAG-VCP was purified by anti-FLAG affinity beads and then subjected to non-reducing SDS-PAGE followed by Ruby staining (Ruby stain) and autoradiography. The amounts of incorporated radioactivity were quantified by BAS5000 (Fuji Film) and were compared with the amount incorporated into VCP in the presence of cycloheximide alone (lane 2) as a reference. Dithiolate treatment induced incorporation of [35S]cysteine into VCP, and this incorporation was inhibited by strongly inactivated VCPs with a high concentration of H2O2 and diamide. E, diamide treatment induced incorporation of [35S]cysteine into VCP, and this incorporation was inhibited in the presence of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis. DTT removed the incorporated [35S]cysteine. D and E, VCP ATPase activity was inhibited by H2O2 and diamide in a dose-dependent manner. The mean values of three independent experiments are shown, where the value of the non-treated VCP serves as the reference value (100%). Bars, S.D.

Flow Cytometric Analysis of Yeasts—After adjustment of the A600 to 0.3, yeast strains grown in the synthetic dropout (SD) medium were treated with diamide at 30 °C for 12 h. Each sample was then fixed in 70% ethanol, resuspended in a Tris-citrate buffer (180 mM Tris-HCl (pH 7.5), 180 mM NaCl, 70 mM MgCl2, 50 mM sodium citrate), briefly sonicated, and digested with 0.25 mg/ml RNase in the same buffer followed by the proteinase K treatment (1 mg/ml), each for an hour at 50 °C. DNA was stained with 50 μg/ml propidium iodide, and 50,000 cells from each sample were scanned with a FACSscan flow cytometer (BD Biosciences) as described previously (37).

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

RESULTS

VCP Is Modified by S-Glutathionylation in Response to Oxidative Stress—We found VCP was modified by DTT-sensitive S-glutathionylation after the treatment with diamide plus GSH, GSNO, or GSNO in vitro (Fig. 1A). Recombinant VCP was treated with diamide plus GSH, GSNO, or GSNO, subjected to non-reducing SDS-PAGE, and detected by Western blot analysis with an anti-GSH antibody (Fig. 1A). When

nm [35S]cysteine (1000 Ci/mmol) at 37 °C for 4 h. Then the cells were washed 3 times with serum-free Dulbecco’s modified Eagle’s medium and treated with or without 5 mM diamide in serum-free media at 37 °C for 10 min. The cells were then washed with ice-cold phosphate-buffered saline and lysed with Triton lysis buffer containing either 50 mM NEM or 25 mM DTT; NEM was used to modify free sulfhydryls to prevent scrambling of the label in the lysates by thiol-disulfide exchange; DTT was used to reverse protein35S-labeled thiolation so as to distinguish the DTT-sensitive post-translational modifications from the cysteine incorporation in the protein backbone. After FLAG-VCP had been purified from lysates based on the above methods, VCP was subjected to non-reducing 7.5% SDS-PAGE (300 ng of VCP/lane) and detected by autoradiography.

Yeast Strains—The genotype of yeast strains are: W303, MATa can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ural-1; Y529, MATa Δcdc48:His3 ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 E305(pRS314/CDC48); Y530, MATa Δcdc48:His3 ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 E307(pRS314/cdc48/T532C). Yeasts were grown in YPAD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose, and 0.004% adenine with or without 2% agar) or in synthetic complete medium (0.67% yeast nitrogen base and 2% glucose supplemented with amino acids and nucleotide bases with or without 2% agar).

Growth Assay of Yeasts—Plates that contained oxidants were prepared a day before use and stored overnight at 4 °C in the dark. The spot assay was performed as described previously; after adjustment of the A600 at 1.0, yeasts were serially diluted from 10⁻¹ up to 10⁻⁵, and 5 μl of each diluted sample was spotted on the plates (approximately, 10,000, 1,000, 100, 10 cells per spot) (36). The yeasts on paraquat plates were incubated for 6 days, and those on the other plates were incubated for 3 days, and then their photographs were taken.
VCP was preincubated with GSH or diamide alone, no bands were detected with the anti-GSH antibody, and VCP was detected at a position corresponding to 97 kDa. However, when VCP was preincubated with diamide plus GSH, GSSG, or GSNO, VCP was detected with the anti-GSH antibody, and its migration positions shifted to the upper positions. S-Glutathionylation is a covalent but reversible modification of cysteine residues with GSH and is removed by DTT treatment (38). After this DTT treatment, the bands detected by the antibody actually disappeared, and their migration positions were restored to those corresponding to 97 kDa (Fig. 1A). Moreover, we were able to detect S-glutathionylation of VCP using non-reducing SDS-PAGE followed by autoradiography. [35S]GSH signals were detected on VCP when VCP was treated with diamide plus [35S]GSH, but after DTT treatment, these [35S]GSH signals disappeared (Fig. 1B). These results clearly demonstrate that VCP can be modified reversibly by GSH in vitro.

Next, we examined whether VCP can be modified by GSH in cultured cells. HEK293T cells were transfected with a FLAG-VCP expression vector and were metabolically labeled with [35S]cysteine in the presence of cycloheximide; in this condition most [35S]cysteines have been shown to be used for GSH synthesis (38). Then these cells were treated with diamide, and FLAG-VCP was immunoprecipitated with an anti-FLAG affinity bead and subjected to non-reducing SDS-PAGE followed by autoradiography (Fig. 1C). This experiment clearly showed that diamide treatment induced incorporation of [35S]cysteine into VCP. This incorporation was inhibited in the presence of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis (Fig. 1C). Furthermore, treatment of VCP with DTT before electrophoresis removed the incorporated [35S]cysteine (Fig. 1C). These results clearly demonstrate that oxidative stress induces S-glutathionylation of VCP in cultured cells.

**Reversible Inactivation of VCP by Oxidative Stress in Vitro**—We next investigated the effect of oxidative stress on VCP ATPase activity. Oxidants such as H2O2 and diamide, a thiol-specific oxidant, were shown to inhibit the ATPase activity of recombinant VCP in a dose-dependent manner (Fig. 1, D and E). Complete inhibition of VCP ATPase activity required 10 mM H2O2 or 100 μM diamide. The inactivation of VCP by H2O2 and diamide were recovered by subsequent DTT treatment (Fig. 1, F and G). However, high concentrations of H2O2 and diamide irreversibly inactivated VCP (Fig. 1, F and G). DTT enhanced the ATPase activity of VCP even in the absence of oxidizing agents, probably due to the reduction of certain oxidations, which might occur naturally or during the purification. Because both diamide and DTT are known to function specifically against thiols (38), these results suggest that oxidative modification of a cysteine residue(s) in VCP would be responsible for the decrease in ATPase activity observed.

**Identification of Oxidized Cysteine Residues of VCP by LC/MS**—To identify the modified sites of VCP in response to oxidative stress, we performed tryptic digestion of oxidant-treated VCP and analyzed its fragments by LC/MS. To eliminate the artificial modification of cysteine residues during sample preparation, we blocked the unmodified cysteine residues with IAA, a thiol-modifying agent that induces carboxymethylation of non-modified, in other words, reduced cysteine residues. When VCP was reduced completely by DTT followed by IAA treatment, 7 carboxymethylated cysteine residues were identified as Cys-69, Cys-77, Cys-105, Cys-535, Cys-572, and Cys-691. For example, a tryptic digestion fragment containing Cys-522 (GVLFYGP-PGGCGK) showed signs of being modified by carboxymethylation. The difference in mass score between b9 and b10 ions was 151 Da, corresponding to an increase of 58 Da, indicative of the sulfonic acid modification of Cys-522 (C). These results indicate that Cys-69, Cys-77, and Cys-522 were capable of being carboxymethylated even in the absence of oxidizing agents, probably due to the reduction of certain oxidations, which might occur naturally or during the purification. Because both diamide and DTT are known to function specifically against thiols (38), these results suggest that oxidative modification of a cysteine residue(s) in VCP would be responsible for the decrease in ATPase activity observed.

**Identification of Oxidized Cysteine Residues of VCP by LC/MS**—To identify the modified sites of VCP in response to oxidative stress, we performed tryptic digestion of oxidant-treated VCP and analyzed its fragments by LC/MS. To eliminate the artificial modification of cysteine residues during sample preparation, we blocked the unmodified cysteine residues with IAA, a thiol-modifying agent that induces carboxymethylation of non-modified, in other words, reduced cysteine residues. When VCP was reduced completely by DTT followed by IAA treatment, 7 carboxymethylated cysteine residues were identified as Cys-69, Cys-77, Cys-105, Cys-535, Cys-572, and Cys-691. For example, a tryptic digestion fragment containing Cys-522 (GVLFYGP-PGGCGK) showed signs of being modified by carboxymethylation. The difference in mass score between b9 and b10 ions was 151 Da, corresponding to an increase of 58 Da (Fig. 2A and not shown). This observation indicated that carboxymethylation of Cys-522 had taken place.

When VCP was treated with IAA after incubation with H2O2 or diamide, Cys-105, Cys-535, Cys-572, and Cys-691 were still identified as carboxymethylated cysteine residues, indicating that Cys-105, Cys-535, Cys-572, and Cys-691 were capable of being carboxymethylated even after treatment with H2O2 or diamide. In contrast, Cys-69, Cys-77, and Cys-522 were not detected as carboxymethylated residues after treatment with these oxidants. These results indicate that Cys-69, Cys-77, and Cys-522 are oxidized by these oxidants. To confirm whether Cys-69, Cys-77, and Cys-522 had been oxidized, we analyzed VCP after diamide treatment by means of LC/MS. We were in fact able to establish an ~48-Da increase of Cys-69, Cys-77, and Cys-522 (Fig. 2B and not shown), indicating that sulfonic acid modification of these cysteines had occurred. The sulfonic acid form of cysteine residues is an irreversible modification known to be converted from S-thiolation (39, 40). These
results indicate that oxidative stress actually induces S-thiolation of Cys-69, Cys-77, and Cys-522 in VCP (Fig. 2C).

**Identification of Cys-522 as a Key Residue for Oxidative Regulation of VCP ATPase Activity**—To identify the regulatory cysteine residue responsible for the decrease of the ATPase activity, we prepared recombinant mutant VCPs, each of which contained a single amino acid substitution on one of the three cysteines. These mutated VCPs are referred to as VCP(C69A), VCP(C77A), and VCP(C522T) and were analyzed for their sensitivities against oxidative stress. All mutated VCPs had ATPase activities comparable with that of the wild-type VCP (Fig. 3A). Among the three, only VCP(C522T) showed a resistance to both H2O2 and diamide (Fig. 3, B and C). Note that VCP(C522A) showed a resistance to these oxidants similar to VCP(C522T) (data not shown).

The ATPase Activity of VCP(C522T) Is Resistant Not Only to ROS but Also to RNS—We originally used H2O2 and diamide as reagents for the induction of oxidative stress. Oxidative stress is induced not only by ROS but also by RNS (23). We then examined the sensitivity of VCPs to RNS. NOR3 and NOC5, nitric oxide donors, reduced the ATPase activity of recombinant wild-type VCP in a dose-dependent manner (Fig. 3D). Moreover SIN1, which produces peroxynitrite (ONOO-), also reduced wild-type VCP ATPase activity (Fig. 3E). Because GSH is most abundant in mammalian cells, ROS and RNS are readily reacted with GSH and are converted to GSSG and GSNO, respectively (23). Accordingly, GSSG and GSNO also inhibited wild-type VCP ATPase activity (Fig. 3F). Surprisingly, VCP(C522T) was resistant to all RNS tested (Fig. 3, D–F). These results led us to the supposition that VCP ATPase activity may be regulated by the modification of Cys-522 under oxidative stress including ROS and RNS as well as NEM.

**Reversible Inactivation of VCP by Oxidative Stress in Cultured Cells**—We next examined the regulatory role of Cys-522 on the ATPase activity in vivo. FLAG-tagged wild-type VCP and VCP(C522T) were expressed in HEK293T cells and were immunopurified after treatment of the cells with diamide. In non-denatured PAGE, VCPs immunopurified from cells treated with diamide (at least up to 10 mM) migrated at a hexamer position (data not shown). Diamide treatment of the cells decreased wild-type VCP ATPase activity in a dose-dependent manner, and this inactivation was recovered by subsequent DTT treatment (Fig. 4A). In accordance with our in vitro experiments, the ATPase activity of VCP(C522T) was not decreased after diamide treatment of the cells. Rather, this activity appeared to be enhanced by diamide treatment (Fig.

---

**FIGURE 3.** The ATPase activity of VCP was regulated via oxidative modulation of only one cysteine residue. A, recombinant mutant VCPs (VCP(C69A), VCP(C77A), and VCP(C522T)) purified from HEK293F cells had comparable ATPase activities to that of recombinant wild-type (WT) VCP. Mean values of triplicate experiments are shown. Bars, S.D. B and C, among them only VCP(C522T) showed a resistance to H2O2 and diamide. D, NOR3 and NOC5 inactivated wild-type VCP ATPase activity, but VCP(C522T) showed a resistance to the inactivation. E, SIN1, a peroxynitrite producer, inactivated wild-type VCP ATPase activity, but VCP(C522T) showed a resistance to the inactivation. F, GSSG and GSNO inactivated wild-type VCP ATPase activity, but VCP(C522T) showed resistance to the inactivation. G, NEM inactivated wild-type VCP ATPase activity, but VCP(C522T) showed resistance to the inactivation. B–G, mean values of three independent experiments are shown, with the value of the non-treated VCP used as the reference value (100%). Bars, S.D.
FIGURE 4. Reversible inactivation of VCP by oxidative stress in cultured cells. A, diamide inhibited VCP ATPase activity in a dose-dependent manner in cultured HEK293T cells. FLAG-VCP-expressing cells were treated with diamide, and FLAG-VCP was purified with anti-FLAG affinity beads, and its ATPase activity was measured. The diamide-decreased ATPase activities were recovered by subsequent DTT treatment. Purified VCP proteins were quantified by Western blot in the presence of β-mercaptoethanol. B, the ATPase activity of VCP(C522T) was not decreased by oxidative stress in HEK293T cells. Note that ATPase activity of VCP(C522T) was in fact increased by diamide treatment and was actually recovered to its original level by subsequent DTT treatment. Purified VCP proteins were quantified by Western blot in the presence of β-mercaptoethanol. WT, wild type. C, Cys-77 but not Cys-522 was modified by S-glutathionylation in HEK293T cells. HEK293T cells were transfected with FLAG-VCP expression vectors, and the cells were incubated with [35S]cysteine in the presence of cycloheximide followed by diamide treatment. After diamide treatment, FLAG-VCPs were purified, and the amounts of incorporated [35S]cysteine were compared. *, p < 0.05; **, p < 0.01. C–C, mean values of three independent experiments are shown, with the value of the non-treated VCP used as the reference value (100%). Bars, S.D. D, Western blot analysis of p47, Npl4, and Ufd1 co-immunoprecipitated (IP) with FLAG-VCP. Diamide treatment did not increase the amount of these VCP partners but increased the binding of Npl4 and Ufd1, but not p47, to VCPs.

Although the mechanism of this up-regulation of VCP(C522T) ATPase activity remains unclear, the activity did recover to its original level by subsequent DTT treatment (Fig. 4B).

We next examined whether Cys-522 can be S-glutathionylated in cultured cells. HEK293T cells expressing the wild-type and mutated FLAG-VCPs were labeled with [35S]cysteine and treated with diamide. Then FLAG-VCPs were immunoprecipitated, and the incorporated radioactivities were measured. [35S]Cysteine was incorporated into FLAG-VCP(C522T) at the same level as wild-type FLAG-VCP, but the amount of the incorporated [35S]cysteine into FLAG-VCP(C77A) was significantly decreased (Fig. 4C). These results indicate that Cys-77 but not Cys-522 is an S-glutathionylation site in cultured cells. However, FLAG-VCP(C77A) was still labeled with [35S]cysteine, suggesting that VCP has another S-glutathionylation site (Fig. 4C). Using LC/MS, we identified the sulfonic acid forms as Cys-69, Cys-77, and Cys-522 after diamide treatment (Fig. 4B). The sulfonic acid form of the cysteine residues is the end product of S-thiolation, such as S-glutathionylation, S-nitrosylation, or disulfide bond formation (39, 40). Diamide has been shown to induce disulfide bond formation in cultured cells (42). Thus, after oxidation by diamide, VCP may form a disulfide bond between Cys-522 and a yet unidentified cysteine residue, resulting in the inactivation of its ATPase.

We then examined whether diamide treatment affects the interaction of VCP to its known binding partners, such as p47, Npl4, and Ufd1 (17). Diamide treatment of the cells did not change the expression levels of these proteins but dose-dependently increased the amounts of Npl4 and Ufd1 that were co-immunoprecipitated with FLAG-VCP (Fig. 4D). The increase in the interaction was also observed with FLAG-VCP(C522T) (data not shown). These results demonstrate that oxidative stress regulates both the ATPase activity and the binding properties of VCP in cultured cells.

The Oxidative Modulation of Cys-522 Regulated ERAD—Several mutations have been identified in spastin, another member of the AAA ATPases, which are responsible for autosomal dominant hereditary spastic paraplegia, another inherited neurodegenerative disorder (43). Among them, two mutations have been found in the Walker A motif of spastin; one was substituted with lysine from asparagine at the corresponding position of Cys-522 of VCP, and the other was substituted with arginine from lysine at the corresponding position of Lys-524 of VCP (44). We, thus, introduced the amino acid substitution on Cys-522 to lysine and found that this mutant, VCP(C522K), almost completely lost its ATPase activity, similar to VCP(K524A) (17) (Fig. 5A). Moreover, VCP(C522K) expression induced phenotypes indistinguishable from those induced by VCP(K524A) expression in cultured cells (17), namely vacuole formation (Fig. 5B), accumulation of polyubiquitinated proteins, induced expression of CHOP, an ER stress marker protein (Fig. 5C), and increased aggregate formation of CFTR(ΔF508), a well known substrate of ERAD (Fig. 5D).

Diamide Induced Cyttoplasmic Vacularization in Cells Expressing CFTR(ΔF508)—Diamide has been reported to induce ER-derived cytoplasmic vacularization followed by cell death in cultured cells (45, 46). These phenotypes were quite similar to those induced by VCP(K524A) and VCP(C522K) (see above paragraph). Hence, we next examined whether Cys-522 of VCP is the target of diamide for the phenotypes.

Contrary to the report (45, 46), we were not able to observe any clear vacularization in all tested cultured cells. However, when VCP was loaded with strong burdens, namely concomitant expressions of CFTR(ΔF508), a VCP substrate in ERAD (17, 47), diamide treatment clearly induced vacularization in the HEK293T cells (Fig. 6A). The overexpression of wild-type VCP further enhanced vacularization in the CFTR(ΔF508)-expressing cells treated with diamide. The reason for this enhancement is currently unknown; overexpression of VCP may sensitize the cells against oxidative stresses. In contrast, any enhancement of the vacuole formation was not observed in the overexpression of VCP(C522T) (Fig. 6B). These results suggest that oxidative stress-induced ER dysfunctions such as cytoplasmic vacularization are mediated via the modulation of Cys-522 of VCP (see “Discussion”).
Cys-522 is Conserved among Multicellular Organisms but Not Unicellular Organisms—Cys-522 is located in the center of Walker A motif of the second ATPase domain, which is responsible for major VCP ATPase activity (17). Cys-522 is evolutionally conserved in multicellular organisms from Caenorhabditis elegans to Homo sapiens but not in unicellular organisms such as yeast Saccharomyces cerevisiae and archaeabacteria Thermoplasma acidophilum (Fig. 7A). At the corresponding position of Cys-522, the yeast homologue Cdc48p has a threonine residue, and the archaeabacteria homologue VCP-like ATPase of Thermoplasma acidophilum (VAT) has a valine residue (Fig. 7A). Therefore, we expected that Cdc48p would be less sensitive to oxidative stress than mammalian VCP. Indeed, the ATPase activity of Cdc48p was not reduced by H$_2$O$_2$ and diamide, as compared with mammalian VCP (Fig. 7B). Cdc48p was also less sensitive to NEM than mammalian VCP (data not shown).

To address in vivo roles of the oxidative modulation of VCP, we used S. cerevisiae as a model system. In yeast, Cys-522 in VCP corresponds to Thr-532 in Cdc48p, and temperature-sensitive cdc48 mutants showed cell growth arrest at the G$_2$/M phase and cell death at restrictive temperatures (48–50). We then constructed mutant strains Y529 (Δcdc48 + CDC48) and Y530 (Δcdc48 + cdc48[T532C]). Y529 and Y530 grew equally well at 30 °C, indistinguishably from a wild-type strain (W303) (Fig. 7C). Western blot analysis showed that equivalent amounts of Cdc48p were expressed in all the strains (Fig. 7C). We then grew these strains in the presence of various oxidants and compared their growth with the spot assay. Of them, only Y530 exhibited hypersensitivities to all the oxidants tested, e.g. H$_2$O$_2$, diamide, NEM, and paraglate (Fig. 7C).
phase in the presence of oxidants, representing dead cells with DNA fragmentation and G2/M arrest cells, respectively, as compared with W303 and Y529 (Fig. 7D). These results collectively demonstrate that oxidation of Cys-522 is crucially important in the regulation of VCP ATPase activity.

**DISCUSSION**

This paper provides a clear demonstration that the ATPase activity of VCP is negatively regulated by oxidative stress. It is notable that this apparently important regulation is performed by the oxidation of a single cysteine residue. Thus, the ATPase activity of VCP was decreased by a variety of oxidative reagents such as oxygen and nitrogen (Figs. 1 and 3). Oxidative stress modified at least three cysteine residues of VCP, Cys-69, Cys-77, and Cys-522 (Fig. 2). Among them, only Cys-522 was found to be responsible for the oxidative regulation of VCP ATPase activity (Fig. 3).

VCP is conserved among the various species to an extraordinary extent, and its amino acid sequences are 100% identical among human, rat, and mouse. Cys-522 is located in the second ATPase domain, which is responsible for the major ATPase activity, and is well conserved among multicellular but not unicellular organisms (Fig. 7A). In Cdc48p (yeast VCP), the amino acid corresponding to Cys-522 is threonine. This amino acid substitution in mammalian VCP, which we referred to as VCP(C522T), retained ATPase activities comparable with that of the wild type (Fig. 3). Interestingly, VCP(C522T) gained almost complete resistance against all oxidative stress tested (Figs. 3 and 4). Consistent with this, Cdc48p showed a similar resistance to oxidative stress as observed in VCP(C522T) (Fig. 7B). It is noteworthy that asparagine at
Red-ox Regulation of VCP ATPase Activity

...the Cys-522-equivalent position in spastin, another AAA ATPase, is mutated to lysine in patients suffering dominantly inherited familial spastic paraplegia, another neurodegenerative disorder (43, 44). Indeed, VCP(C522K) lost its ATPase activities (Fig. 5A). These results demonstrate the importance of this cysteine residue within the Walker A motif in the regulation of VCP ATPase activity, and oxidized cysteine and lysine at this position probably interfere with ATP to bind to VCP through the mechanism of steric hindrance.

Several AAA proteins have cysteine residues at the Cys-522-equivalent positions in Walker A motif (Fig. 7A). Most of them, including VCP, are reportedly inactivated with regard to their ATPase activities by nitric oxide (NO) (45). NO is one of the most important factors in cell-cell communication (33). Obviously, ROS and RNS are also expected to be involved in such roles.

We detected Cys-77 as a modification site of S-glutathionylation (Fig. 4C). Cys-77 is surrounded by acidic and basic residues. The cysteine residue in such an environment is modified easily by both S-glutathionylation and S-nitrosylation (57). It has been suggested that VCP has another S-glutathionylation site (Fig. 4C). Cys-69 would be the site because the environment of Cys-69 is similar to Cys-77. Both Cys-69 and Cys-77 are located in its N domain, and this domain regulates the binding properties of VCP (11). Therefore, oxidative stress may also contribute to regulate the binding properties of VCP to its partner via the oxidative modification of Cys-69 and/or Cys-77. Although this idea remains to be verified, diamide treatment of cultured cells actually strengthened VCP interaction with Npl4 and Ufd1 (Fig. 4D), which may further contribute to ERAD inhibition.

Oxidative stress has been suspected to be involved in the pathological processes of several human neurodegenerative disorders, especially Parkinson disease (see the Introduction). However, no target molecule of oxidative stress has as yet been identified in such pathological processes. Recent studies have suggested that ERAD inhibition and ER stress play important roles in the pathology of several neurodegenerative disorders such as polyglutamine diseases (58, 59), Parkinson disease (60, 61), and Alzheimer disease (62–64), etc. Our present study together with these observations strongly indicates that oxidative modulation of VCP is a crucial link between oxidative stress and ERAD inhibition as well as ER stress in neurodegenerative disorders. Accumulation of abnormal proteins is another common hallmark well recognized in the pathology of neurodegeneration (1, 2). Several recent reports have indicated that accumulation of abnormal proteins can produce oxidative stress (65, 66). Indeed, we observed that accumulation of expanded polyglutamines induced fluorescence of ROS-sensitive dyes such as 5-(and-6)-chloromethyl-2′,7’-dichlorodihydrofluorescein diacetate, acetylated ester. Oxidative stress increases as animals grow older (23). In addition to oxidative stress by accumulation of abnormal proteins, such aging-related oxidative stress may result in further inactivation of VCP via Cys-522 modification. This might be a reason why many neurodegenerative disorders occur after middle age.

In yeast, complementation of the endogenous CDC48 gene deletion by wild-type CDC48 or cdc48[T532C] was successfully achieved; these mutant strains were referred to as Y529 or Y530, respectively. Indeed, Y530 showed higher sensitivities to several oxidants such as H2O2, diamide, NO, and paraquat in the spot assay (36), and showed DNA fragmentation and G2/M arrest after exposure to these oxidants (Fig. 7, C and D). Both phenotypes are indeed those of temperature-sensitive mutants of cdc48 (data not shown) (48–50). These results clearly demonstrated that at least in yeast ATPase activities of Cdc48p(T532C) are regulated by several oxidants in vivo. We vigorously attempted to show that overexpression of VCP(C522T) renders mammalian cells resistant to certain stresses, but we could not obtain substantial evidence apart from the suppression of diamide-induced vacuole formation. This may be due to the presence of endogenous VCP; the complete replacement or complete knock-down of endogenous VCP may be necessary to obtain clear phenotypes, as observed in the yeast experiments.

In summary, our results shed light on the regulatory mechanism of VCP ATPase activity in response to oxidative stress. This mechanism may be important in certain physiological conditions such as in protection to oxidative stress as well as in the pathogenesis of certain disorders such as neurodegenerative disorders. Further molecular analysis of this...
regulatory mechanism of VCP may well provide some clues for the interpretation of the general mechanisms of neurodegeneration and for curing or preventing neurodegenerative disorders that are as yet untreatable.

Acknowledgments—We thank K. Kitagawa and K. Kuroiwa for technical assistance, M. Sagimoto for secretarial assistance, and our laboratory members for valuable discussions. We also thank R. R. Kopito for a GFP-CFTR(ΔF508) expression vector.

REFERENCES

1. Kakizuka, A. (1998) Trends Genet. 14, 396–402
2. Kobayashi, T., and Kakizuka, A. (2003) Cytogenet. Genome Res. 100, 261–275
3. Kimura, Y., and Kakizuka, A. (2003) JIBMB Life Mag. 55, 337–345
4. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) Science 292, 1552–1555
5. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) Nature 392, 605–608
6. Shimura, H., Hattori, N., Kudo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwaya, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) Nat. Genet. 25, 302–305
7. Leroy, E., Boyer, R., Auburger, G., Lebe, U., Ulm, G., Mege, E., Hatta, G., Brownstein, M. J., Jonnalagada, S., Chernova, T., Deheja, A., Lavedan, C., Gasser, T., Steinbach, P. J., Wilkinson, K. D., and Polymèrespol, M. H. (1998) Nature 395, 451–452
8. Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996) Nat. Genet. 13, 196–202
9. Hirabayashi, M., Inoue, K., Tanaka, K., Nakadate, K., Ohswa, Y., Kamei, Y., Pospie, A. H., Sinohara, A., Iwamatsu, A., Kimura, Y., Uchiyama, Y., Hori, S., and Kakizuka, A. (2001) Cell Death Differ. 8, 977–984
10. Kawaguchi, Y., Okamoto, T., Yamauchi, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, N., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S., and Kakizuka, A. (1994) Nat. Genet. 8, 221–228
11. Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokunor, P., Lilly, J., Leonard, G., Meyer, H., van Heel, M., and Freemont, P. S. (2000) Mol Cell. Biol. 6, 1473–1484
12. Wojcik, C., Yano, M., and DeMartino, G. N. (2004) J. Cell Sci. 117, 281–292
13. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Whyte, M. P., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., and Botstein, D. (2002) Hum. Mol. Genet. 11, 153–163
14. Ueda, S., Nakamura, H., Masutani, H., Sasa, S., Yonehara, S., Takahayashi, A., Yamanaka, Y., and Yodoi, J. (1998) J. Immunol. 161, 6689–6695
15. Ault, G., and Lawrence, D. A. (2003) Exp. Cell Res. 285, 9–14
16. Gnann, A., Rioran, J. R., and Wolf, D. H. (2004) Mol. Cell. Biol. 15, 4125–4135
17. Moir, D., Stewart, S. E., Osmond, B. C., and Botstein, D. (1982) Genetics 100, 547–563
18. Fu, X., Ng, C., Feng, D., and Liang, C. (2003) J. Cell Biol. 163, 21–26
19. Frohlich, K. U., Fries, H. W., Rudiger, M., Erdmann, R., Botstein, D., and Mecke, D. (1999) Cell 104, 443–453
20. Zhang, L., Ashendel, C. L., Becker, G. W., and Morre, D. J. (1994) J. Cell Biol. 127, 1871–1883
21. Peters, J. M., Walsh, M. J., and Franke, W. W. (1990) EMBO J. 9, 1757–1767
22. Matsushita, K., Morrell, C. N., Mason, R. J., Yamakuchi, M., Khanday, F. A., Irani, K., and Lowenstein, C. J. (2005) J. Cell Biol. 170, 73–79
23. Rutkowski, D. T., and Kaufman, R. J. (2003) Dev. Cell 4, 442–444
24. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Liu, D. C., Calton, M., Sardi, N., Yun, C., Popko, B., Pales, R., Stoïj, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) Mol. Cell 11, 619–633
25. Zaman, K., McPherson, M., Vaughan, J., Hunt, J., Mendes, F., Gaston, B., and Palmer, L. A. (2001) Biochem. Biophys. Res. Commun. 284, 65–70
26. Perez-Mato, I., Castro, C., Ruiz, F. A., Corrales, F. J., and Mato, J. M. (1999) J. Cell Biol. 147, 17075–17079
27. Kouroukis, Y., Fujita, E., Jimbo, A., Kikuchi, T., Yamagata, T., Momoi, M. Y., Komi- nami, E., Kuida, K., Sakamaki, K., Yonehara, S., and Momoi, T. (2002) Hum. Mol. Genet. 11, 1505–1515
28. Hishitoh, H., Matusawa, A., Tomiyama, K., Kuida, K., Inoue, H., Morisita, K., and Ichijo, H. (2002) Genes Dev. 16, 1345–1355
29. Imai, Y., Soda, M., and Takahashi, R. (2000) J. Biol. Chem. 275, 35661–35664
30. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001) Cell 105, 891–902
31. Sato, N., Imai, K., Manabe, T., Taniguchi, M., Hidaka, H., Katayama, T., Yonezawa, T., Morihara, T., Yoda, Y., Takagi, T., Kudo, T., Tsuda, T., Itoyama, Y., Mikiyuki, T., Fraser, P. E., and Tohyama, M. (2001) Biochem. Biophys. Res. Commun. 284, 9–14
32. Nakagawa, T., Zhu, H., Morishima, N., Ito, Y., Yankner, B. A., and Yuan, J. (2000) Nature 403, 98–103
33. Wytenbach, A., Sauveaguet, O., Carmichael, J., Diaz-Latoud, C., Arroyo, A. P., and Rubenstein, D. C. (2002) Hum. Mol. Genet. 11, 1317–1351
34. Jell, P., and Mouradian, M. M. (2002) Neurosci. Lett. 320, 146–150
35. Seron, R., and Lamas, S. (2000) Eur. J. Biochem. 267, 4928–4944