Protein kinase CK2 regulates the formation and clearance of aggresomes in response to stress

Masahiko Watabe and Toshio Nakaki*

Department of Pharmacology, Teikyo University School of Medicine, Tokyo 173-8605, Japan

*Author for correspondence (nakaki@med.teikyo-u.ac.jp)

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Summary

Misfolded protein aggregates elicit a stress response, and their clearance is crucial for cell survival. These aggregates are transported by cytoplasmic deacetylase HDAC6 and dynein motors to the aggresome via the microtubule network, and are removed by autophagic degradation. HDAC6 activity is necessary for both the transport and clearance of protein aggregates. However, the cellular factors that regulate HDAC6 activity remain unknown. Here we show that protein kinase CK2 is a crucial modulator of HDAC6 activity because CK2 directly phosphorylates HDAC6 and increases cytoplasmic deacetylase activity. Indeed, cells that expressed HDAC6 mutated at Ser458, a CK2-mediated phosphorylation site, failed to both form and clear aggresomes, and increased cytotoxicity. Interestingly, Ser458 is conserved only in higher primates, such as human and chimpanzee, but not in the rhesus macaque. These findings identify CK2 as a crucial protein involved in the formation and clearance of aggresomes, and hence in cell viability in response to misfolded protein stress.

Key words: CK2, Histone deacetylase 6, Aggresome

Introduction

Protein folding is the process by which a linear polymer of amino acids is converted to a unique three-dimensional structure comprising a functional protein molecule. However, failure to correctly translocate and integrate such proteins can result in the formation of misfolded proteins. Misfolded proteins are prone to forming aggregates that interfere with normal cellular function (Plemper and Wolf, 1999) and are closely monitored, processed and degraded to prevent their accumulation in cells. Misfolded proteins are degraded primarily by the proteasome (Kopito, 1997). However, when misfolded proteins are not degraded efficiently by the proteasome machinery, their efficient disposal is essential for cell survival, because aggregates formed by misfolded proteins are toxic (Kopito, 2000). In fact, failure to degrade misfolded protein aggregates is a dominant contributing factor to neuronal cell death in many neurodegenerative diseases such as Parkinson's disease (Thomas et al., 1995).

The recent discovery of the aggresome, as a cellular organelle, provides an important clue to elucidating the molecules in the pathway responsible for the clearance of misfolded protein aggregates. Formation of aggresomes is a specific and active cellular process that has evolved to cope with excessive levels of misfolded protein aggregates (Kopito, 2000). Aggresomes have an interesting clinical aspect in that they are similar to the cytoplasmic inclusion bodies commonly observed in many neurodegenerative diseases. Aggresomes and Lewy bodies, which are the hallmark cytoplasmic inclusion bodies found in neurons affected by Parkinson's disease, share remarkable biochemical and morphological characteristics (McNaught et al., 2002). Therefore, aggresomes are crucial to the understanding of both the misfolded protein-induced stress response and the pathogenesis of neurodegenerative disease. However, little is known about the regulatory mechanism underlying aggresome formation.

Protein kinase CK2 is ubiquitously expressed and highly conserved in eukaryotic cells (Allende and Allende, 1995; Blanquet, 2000; Litchfield, 2003). It comprises two catalytic α- or α′-subunits and two regulatory β-subunits to form a heterotetrameric structure in which two β-subunits dimerize to link the two α- or α′-subunits (Niefind et al., 2001). A number of studies have indicated that CK2 is involved in a wide variety of cellular processes, including the cell cycle, apoptosis, transcriptional regulation and signal transduction (Allende and Allende, 1995; Litchfield, 2003; Meggio and Pinna, 2003; Watabe et al., 1997). CK2 is instrumental to, and necessary for, the promotion of cell survival (Ahmed et al., 2002; Litchfield, 2003). Disruption of the genes encoding both the catalytic subunits of CK2 is synthetically lethal in fission yeast (Padmanabha et al., 1990; Rethinaswamy et al., 1998). Similarly, it is embryonically lethal when CK2β is knocked down in *Caenorhabditis elegans* by RNA interference, or in mice by gene disruption, which is reminiscent of the essential role of CK2β during embryonic development and organogenesis (Buchou et al., 2003; Fraser et al., 2000). Hence, production of both the α- and β-subunits of CK2 appears to be necessary for cell viability.

As a protein serine/threonine kinase, CK2 has a broad substrate spectrum, and over 300 protein substrates of CK2 have been identified to date (Meggio and Pinna, 2003). It is known that one of the CK2 substrates is an intermediate chain of cytoplasmic dynein (Karki et al., 1997). Cytoplasmic dynein is a mechanochemical ATPase that powers retrograde organelle transport in a wide variety of cell types. It has been implicated in many different cellular functions, including the endocytotic pathway, Golgi positioning, chromosome movement, spindle formation, nuclear positioning and retrograde axonal transport. Both the intracellular targeting and the motor activity of cytoplasmic dynein are controlled within the cells. Cytoplasmic dynein is a macromolecular complex of two heavy chains, three intermediate chains and four light chains. The cytoplasmic dynein intermediate

chains have been suggested to be functionally analogous to the axonemal dynein intermediate chains in targeting the dynein motor to its cargo (Thaler and Haimo, 1996). CK2 binds to and phosphorylates its cytoplasmic dynein intermediate chains (Karki et al., 1997). This indicates that CK2 might modulate dynein function at the level of dynein complex assembly, motor activity or specificity of interaction with its cargo. Furthermore, CK2 is also involved in the regulation of microtubule cytoskeleton reorganization. CK2 catalytic α- and α′-subunits bind to tubulin (Faust et al., 1999; Lim et al., 2004b). This binding regulates microtubule dynamics, such as microtubule assembly and stabilization, without the catalytic activity of CK2 kinase (Lim et al., 2004b). Thus, CK2 is a multifunctional protein kinase.

HDAC6 is a unique member of the histone deacetylase family and it deacetylates not only histones (Grozinger et al., 1999) but also α-tubulin (Hubbert et al., 2002), Hsp90 (Kovacs et al., 2005) and cortactin (Zhang et al., 2007). In particular, deacetylation of the Lys40 residue on α-tubulin has been shown to increase microtubule dynamics (Matsuyama et al., 2002; Tran et al., 2007). Deacetylation of α-tubulin might facilitate cellular processes that require microtubule-related transport. Because HDAC6 binds to ubiquitylated proteins through a C-terminal ubiquitin-binding zinc finger domain (UBD), HDAC6 has the potential to be involved, not only in cellular functions depending on the microtubule cytoskeleton, but also in the proteasome-dependent degradation of misfolded proteins. HDAC6 accumulates in aggresome through its interaction with the dynein motor complex and mediates the clearance of misfolded and ubiquitylated proteins (Kawaguchi et al., 2003). Recent studies have shown that HDAC6 mediates the reversible recruitment of aggregated proteins to the centrosome, and that the HDAC6-mediated deacetylation of tubulin is required for accumulation of aggregated proteins (Jiang et al., 2008). HDAC6 also has a role in the eventual clearance of aggresomes, implying a functional connection between HDAC6 and autophagy (Iwata et al., 2005; Lee et al., 2010; Pandey et al., 2007). In particular, HDAC6 controls the fusion of aggresomes to lysosomes by altering the acetylation level of cortactin (Lee et al., 2010), which is the F-actin-binding protein and the substrate of HDAC6 (Zhang et al., 2007), and promotes autophagy in response to misfolded protein stress.

Thus, HDAC6 has a crucial role in the accumulation of misfolded protein aggregates at the aggresomes and the clearance of aggresomes by autophagy. However, little is known about the regulation of HDAC6 deacetylation activity. Here, we provide evidence that CK2 binds directly to HDAC6 and acts as an adaptor for HDAC6 binding to dynein motor proteins without CK2 kinase function. Furthermore, CK2 directly phosphorylates Ser458 on HDAC6. This phosphorylation causes the activation of HDAC6 deacetylase activity, and controls proper aggresome formation and clearance in response to misfolded protein stress.

Results

CK2 directly binds and phosphorylates HDAC6

The association between HDAC6 and CK2 was probed by a pull-down assay using recombinant proteins, GST–HDAC6 and CK2. CK2 was found to directly associate with HDAC6 (Fig. 1A). Primary amino acid sequence analysis of human HDAC6 revealed 26 potential CK2 phosphorylation sites. To investigate whether or not CK2 could phosphorylate HDAC6 in vitro, we incubated GST–HDAC6 with CK2 and [γ-^{32}P]ATP. CK2 caused the phosphorylation of two proteins; one was CK2β, which was formed by autophosphorylation of CK2 itself at Ser2 and Ser3 by the action of the catalytic subunits (Boldyreff et al., 1993; Litchfield et al., 1991); this enhances its stability (Zhang et al., 2002). The other is HDAC6 (Fig. 1B), which indicates that HDAC6 is readily phosphorylated by CK2.

To determine the phosphorylation sites of HDAC6 by CK2, we first performed phosphoamino acid analysis, because the potential CK2 phosphorylation site on HDAC6 includes tyrosine, serine and threonine. Only phosphoserine was detected in CK2-phosphorylated HDAC6 (Fig. 1C). To confirm that CK2 phosphorylates HDAC6 at a serine residue, we performed western blotting analysis using an anti-phosphoserine antibody after incubation of GST–HDAC6 with CK2 in the presence or absence of ATP. CK2 caused the phosphorylation of HDAC6 and CK2β serine residues (Fig. 1D). CK2α was also detected by the anti-phosphoserine antibody and was not phosphorylated by itself; because it was detected in the absence of ATP (Fig. 1D, lane 2), it had been endogenously phosphorylated. This suggests that the phosphorylation of HDAC6 by CK2 occurs at serine residues.

Nine serine residues are potential CK2 phosphorylation sites on human HDAC6. To determine the actual phosphorylation sites of HDAC6 by CK2, we used an auto-spot peptide array technique coupled with a filter-based kinase assay (Schafer et al., 2008). Using this approach with pairs of synthetic wild-type and corresponding alanine mutant 10mer peptides spotted on a cellulose filter as substrates, we obtained only one heavily phosphorylated peptide, with the sequence VEESEEEGPW, from amino acids 455 to 464 (Fig. 1E).

To confirm whether or not CK2 phosphorylated Ser458 of HDAC6 on the full three-dimensional conformation, phosphorylated HDAC6 was subjected to SDS-PAGE. The band obtained was digested with trypsin in-gel and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Fig. 1F). A tryptic fragment with a molecular weight of 3860 Da was detected, and this molecular weight was compatible with the dephosphorylated form (448–480) (3860 Da) (Fig. 1F, left). This fragment peak was decreased upon phosphorylation, and a new peak appeared with a molecular weight of 3941 Da, which is compatible with the phosphorylated form (448–480) (Fig. 1F, right). To determine the exact phosphorylation site, we obtained a tandem mass spectrometry (MS/MS) spectrum of the phosphopeptide (448–480) and identified Ser458 as the phosphorylated residue (Fig. 1G).

CK2 supports binding of HDAC6 and dynein

Interestingly, Ser458 of HDAC6, which is phosphorylated by CK2 is conserved only in higher primates such as human and chimpanzee, and not in mouse, rat, dog, bovine or rhesus macaque (Fig. 2A). This residue also exists in the dynein motor binding domain (Kawaguchi et al., 2003). We incubated GST–HDAC6 with CK2 in the presence or absence of ATP to determine whether the phosphorylation of HDAC6 by CK2 is required for binding of HDAC6 and dynein by immunoprecipitates of endogenous dynein from human SH–SY5Y cells. Surprisingly, even when excess HDAC6 was added to dynein, we detected no binding of HDAC6 and dynein in the absence of CK2 (Fig. 2B, lane 1). However, the addition of CK2 readily induced the binding of HDAC6 and dynein (Fig. 2B, lane 2) and the amount of HDAC6 that bound to dynein showed no change, irrespective of the phosphorylation status of CK2 (Fig. 2B, lanes 2 and 3). This suggests that CK2 is required for efficient binding of HDAC6 and dynein in a phosphorylation-independent manner.

To verify the association of HDAC6 with CK2, we performed a pull-down assay using two recombinant proteins (GST–HDAC6 and CK2 holoenzyme) and individual subunit proteins (Fig. 2C, top). Only CK2 holoenzyme was found to be associated with HDAC6, whereas CK2α and CK2β alone failed to precipitate with HDAC6, indicating that the CK2 holoenzyme associates with HDAC6 (Fig. 2C, bottom). To verify the association of HDAC6 and dynein with CK2 using immunoprecipitates of endogenous dynein brought down from human SH-SY5Y cells, we incubated GST–HDAC6 with CK2 holoenzyme or individual subunit proteins. Only the CK2 holoenzyme was found to associate with dynein; CK2α and CK2β failed to co-immunoprecipitate with dynein, indicating that the CK2 holoenzyme associates with HDAC6 and dynein (Fig. 2D).

HDAC6 phosphorylation by CK2 increases deacetylase activity

To investigate whether the phosphorylation of HDAC6 by CK2 affects deacetylase activity, we used immunoprecipitates of endogenous dynein brought down from human SH-SY5Y cells and incubated GST–HDAC6 with CK2 in the presence or absence of ATP, then measured the deacetylase activity of HDAC6. The phosphorylation of HDAC6 by CK2 increased deacetylase activity (Fig. 2E).

Regulation of tubulin acetylation by CK2 in a rotenone model

We have shown that CK2 supports the binding of HDAC6 to dynein without a catalytic reaction and that the phosphorylation of HDAC6 by CK2 increases deacetylase activity. These observations led us to hypothesize that CK2 functions to load HDAC6 and misfolded proteins onto the dynein motor for transport to aggresomes. If this hypothesis was true, CK2 should be required for the proper formation of aggresomes. We previously investigated neuronal degeneration by using an in vitro rotenone model of human SH-SY5Y cells (Watabe and Nakaki, 2004; Watabe and Nakaki, 2007a; Watabe and Nakaki, 2007b; Watabe and Nakaki, 2008). Rotenone was used to generate oxidative stress in the cells

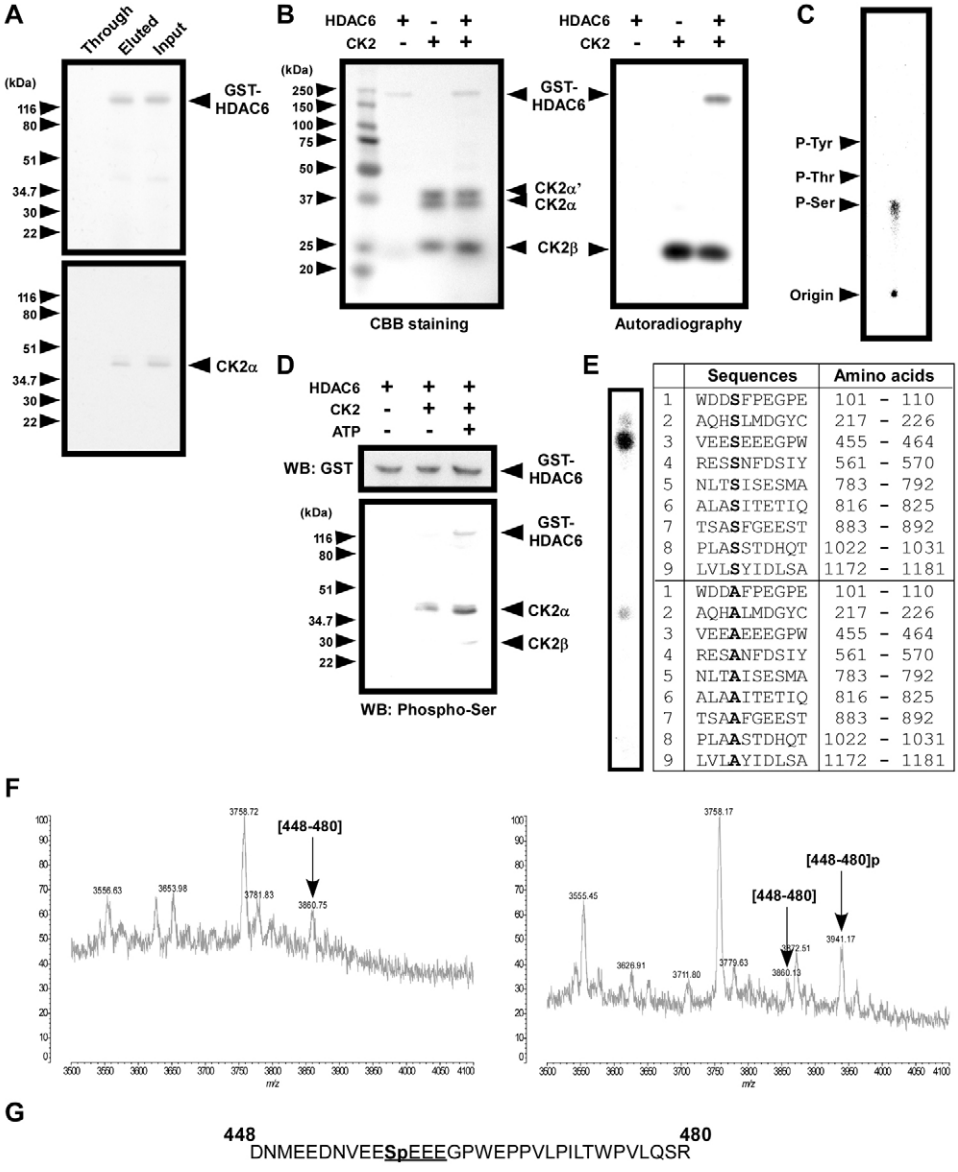

Fig. 1. CK2 directly binds to and phosphorylates HDAC6 at Ser458. (**A**) HDAC6 binds to CK2. Pull-down assay was performed using GST–HDAC6, recombinant CK2 and GSH beads. Bound CK2 was detected by immunoblotting. (**B**) In vitro kinase assay using GST–HDAC6, recombinant CK2 and [γ-^{32}P]ATP. (**C**) ^{32}P-labeled phosphoamino acids from HDAC6 phosphorylated by CK2 separated using thin-layer chromatography. (**D**) Immunoblotting analysis using anti-phosphoserine or anti-GST antibody. (**E**) Ten-residue peptides were synthesized on a cellulose membrane. The membrane was incubated with CK2 and [γ-^{32}P]ATP, and phosphorylated peptides were detected by autoradiography. (**F**) Portion of the MALDI-TOF MS spectrum of HDAC6 phosphorylated by CK2 and digested with trypsin as described in the Materials and Methods. The peaks corresponding to the tryptic peptide (448–480) and its phosphorylated form (448-480)p are indicated. (**G**) Mapping of CK2-phosphorylated site in HDAC6. The CK2 phophorylation motif is underlined.

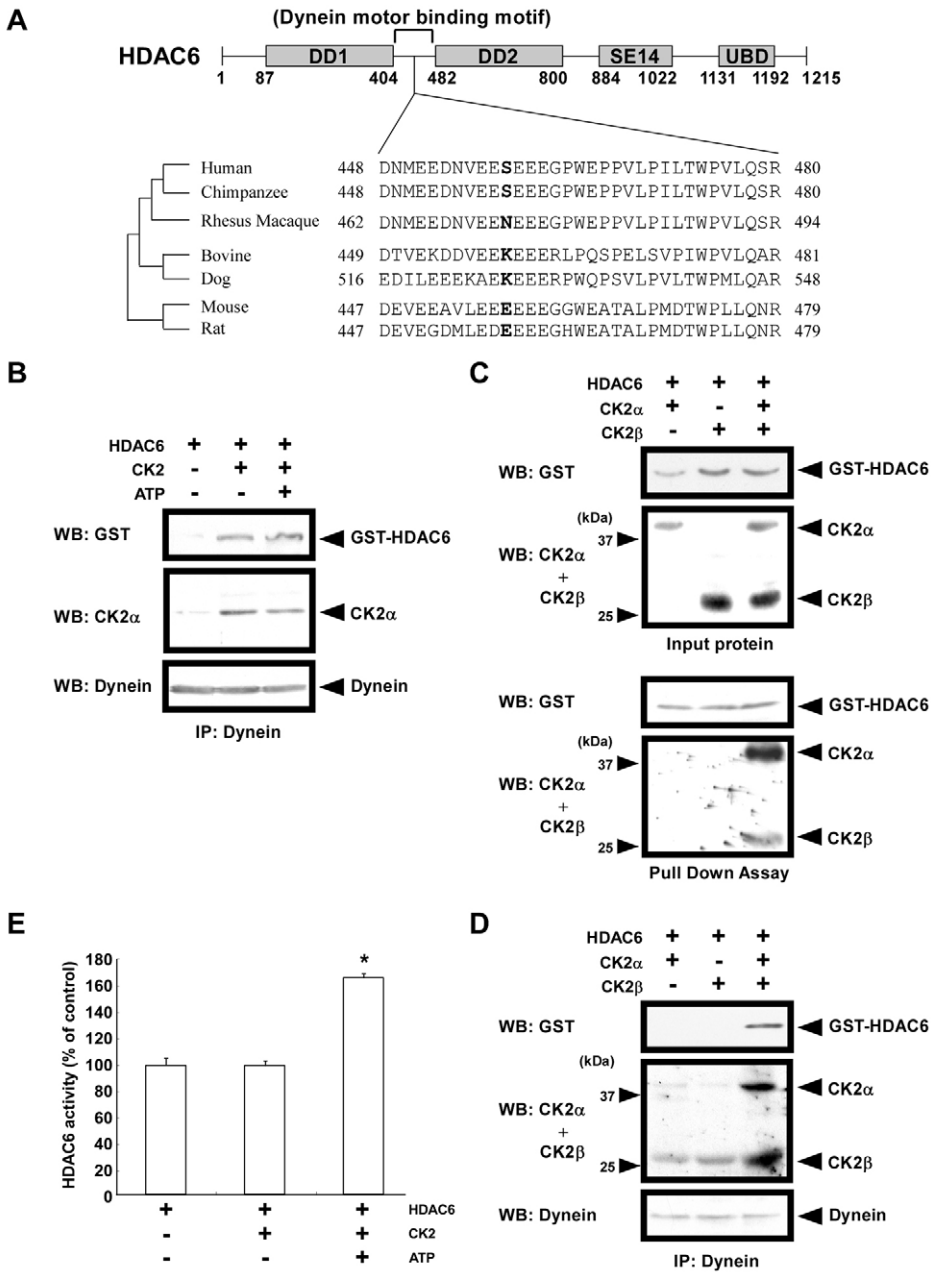

Fig. 2. Binding of HDAC6 and dynein by CK2. (**A**) Phylogenetic tree and partial sequences of CK2-phosphorylated site in HDAC6. (**B**) Immunoblot analysis of SH-SY5Y cell lysates immunoprecipitated with anti-dynein antibody. Samples were incubated with GST–HDAC6 in the presence of recombinant CK2 and ATP and blotted with antibodies against GST, CK2α and dynein. (**C**) Using GST–HDAC6, recombinant CK2α, CK2β and CK2 holoenzyme (top two panels), a pull-down assay was performed (bottom two panels). (**D**) Anti-dynein antibody immunoprecipitates from cell lysates prepared from SH-SY5Y cells were incubated with GST–HDAC6 in the presence of recombinant CK2α, CK2β and CK2 holoenzyme. Immunoblot analysis was performed using antibodies against GST, CK2α, CK2β and dynein. (**E**) HDAC6 activity in the presence or absence of CK2.

and we analyzed aggresome formation. We detected aggregated HDAC6, parkin, dynein and γ-tubulin, which are all components of aggresomes; the intermediate filament protein vimentin redistributed to form a cage that surrounded the pericentriolar core aggregated proteins (Fig. 3). These observations indicate that this model is suitable for aggresome formation analysis.

We examined the change in CK2 concentration with this model. An increase in CK2 concentration was detected in the presence of rotenone, which was not affected by the CK2 inhibitor DRB (Fig. 4A). Using this model, we previously reported that the inhibition of rotenone-generated ROS or NO protected the cells from its toxicity (Watabe and Nakaki, 2007b; Watabe and Nakaki, 2008). Both L-NAME, an inhibitor of nitric oxide synthase (NOS), and N-acetylcysteine (NAC), a scavenger of ROS, suppressed the rotenone-induced increase in CK2 (Fig. 4A). Under these conditions, we examined the acetylated level of tubulin, a cytosolic substrate of HDAC6. Rotenone induced the decrease in acetylated tubulin (Fig. 4A). DRB suppressed the rotenone-induced decrease in acetylated tubulin, but not the rotenone-induced increase in CK2 (Fig. 4A). L-NAME, in combination with NAC, suppressed both the rotenone-induced increase in CK2 and the decrease in acetylated tubulin (Fig. 4A). When the deacetylation activity of HDAC6 was directly measured by immunoprecipitation with an anti-HDAC6 antibody, rotenone increased its activity and this increase was suppressed by DRB and treatment with L-NAME and NAC (Fig. 4B).

Furthermore, we examined the changes in concentration of CK2 and dynein bound to HDAC6 and the phosphorylated levels of HDAC6. Rotenone induced increases in the binding of CK2 and dynein to HDAC6, as well as in phosphorylation at the serine residue of HDAC6 (Fig. 4C). Specificity of these obtained bands was confirmed by a control experiment (supplementary material

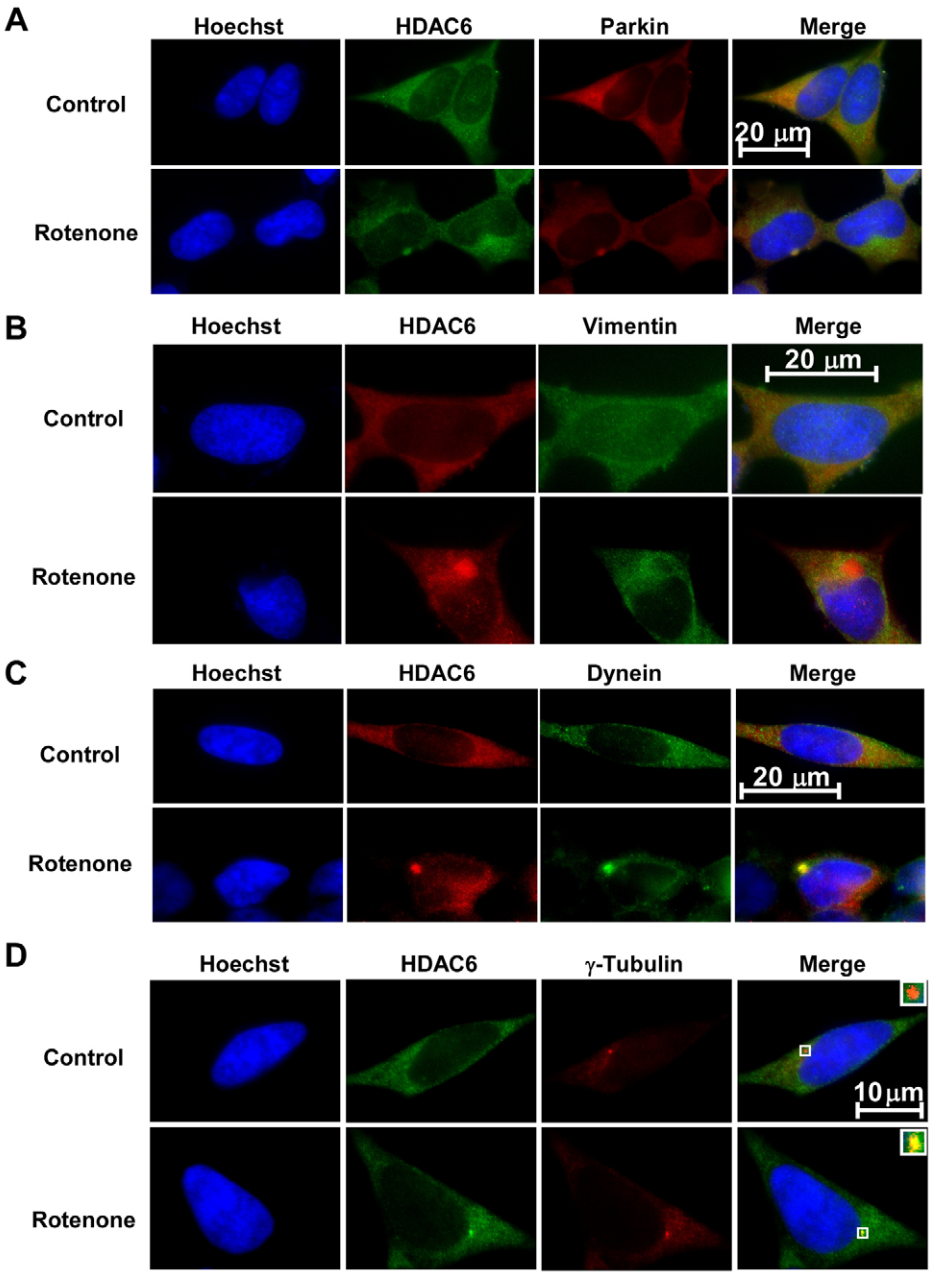

Fig. 3. Detection of aggresome in rotenone-treated SH-SY5Y cells. SH-SY5Y cells were treated with 0.04 μM rotenone for 72 hours. The nuclei were stained with Hoechst (blue) and aggresome-related proteins were immunocytochemically detected using specific antibodies: (**A**) HDAC6 (green) and parkin (red), (**B**) HDAC6 (red) and vimentin (green), (**C**) HDAC6 (red) and dynein (green), (**D**) HDAC6 (green) and γ-tubulin (red).

Fig. S1A). DRB suppressed rotenone-induced HDAC6 phosphorylation but did not increase the binding of CK2 and dynein to HDAC6 (Fig. 4C). Another CK2-specific inhibitor TBB also suppressed rotenone-induced HDAC6 phosphorylation (supplementary material Fig. S1B). Both L-NAME and NAC suppressed the rotenone-induced increase in CK2 binding to HDAC6 and HDAC6 phosphorylation (data not shown). These observations indicate that rotenone causes tubulin deacetylation by HDAC6 phosphorylation via increased binding of CK2 to HDAC6.

Next, we immunocytochemically examined intracellular localization of HDAC6 and the level of tubulin acetylation. Rotenone partially induced a decrease in tubulin acetylation (Fig. 5A), but had no effect on the tubulin concentration (Fig. 5B). Surprisingly, a high concentration of the acetylated form of tubulin was maintained, even at sites where HDAC6 localized in control cells (Fig. 5A,B), indicating that the presence of HDAC6 itself is not sufficient to deacetylate tubulin. Because this result might indicate that the deacetylation activity of HDAC6 is not activated in control cells, we focused on CK2 as a regulator of HDAC6 activity and performed co-staining with an anti-CK2 antibody. CK2 showed the same localization with aggregated HDAC6 upon rotenone treatment (Fig. 5C), and a decrease in levels of tubulin acetylation was detected where CK2 levels were increased (Fig. 5D). These results suggest that HDAC6 is phosphorylated and activated by rotenone-stimulated increases in CK2, and that HDAC6 deacetylates tubulin.

CK2 suppression increases cell toxicity in response to stress

To verify the role of CK2 in the response to stress, we examined the effect of the CK2 inhibitor DRB on cell toxicity using the rotenone model. DRB accelerated rotenone-induced DNA

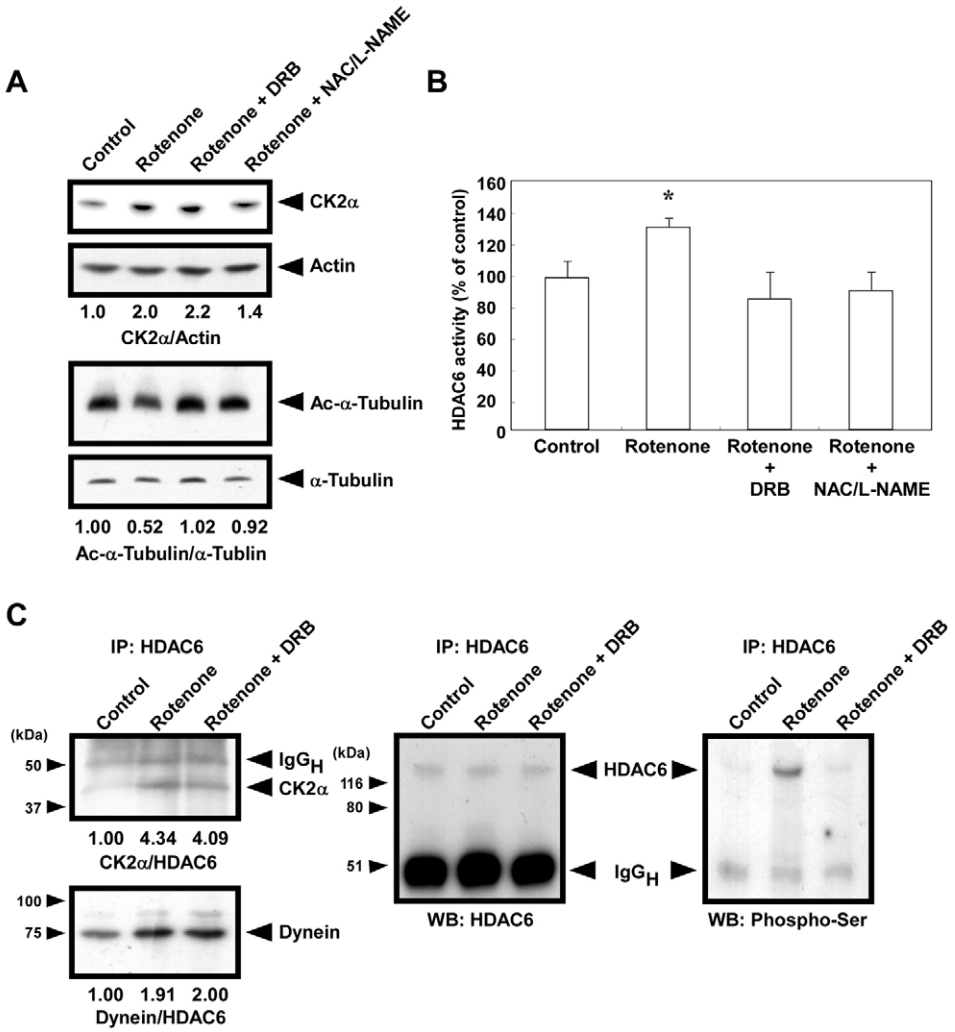

Fig. 4. Induction of CK2 and activation of HDAC6 in rotenone-treated SH-SY5Y cells. SH-SY5Y cells were treated with 0.04 μM rotenone for 72 hours in the presence or absence of 10 μM DRB, 1 mM L-NAME and 3 mM NAC. (**A**) Immunoblot analysis using antibodies against CK2, actin, acetylated α-tubulin and α-tubulin. (**B**) HDAC6 activity in anti-HDAC6 immunoprecipitates and cell lysates. (**C**) Immunoblot analysis using antibodies against CK2α, dynein, HDAC6 and phosphorylated Ser.

fragmentation (Fig. 6A); TBB had the same effect (supplementary material Fig. S1C). To examine the effect of CK2 depletion, we used RNA interference to decrease the CK2 concentration. We analyzed CK2 immunoreactivity immunocytochemically and confirmed a decrease in CK2 in the siRNA-treated cells compared with cells treated with control siRNA (Fig. 6B,C). Rotenone induced nuclear fragmentation in only CK2-knockdown cells and not in the control (Fig. 6B). When we evaluated apoptotic cells by their nuclear morphology (supplementary material Fig. S2), CK2 siRNA quantitatively increased the number of rotenone-induced apoptotic cells compared with levels observed in control cells (Fig. 6C). These results indicate that depletion of CK2 accelerates rotenone-induced cell death without aggresome formation.

Phosphorylation of HDAC6 by CK2 is associated with aggresome formation in response to stress

To investigate whether the phosphorylation of HDAC6 by CK2 is involved in the response to misfolded protein stress, we used two HDAC6 mutants at the CK2 phosphorylation site (Ser 458): a phosphorylation-defective single alanine mutant S458A and a phosphorylation mimic single glutamic acid mutant S458E. First, we suppressed endogenous HDAC6 to reduce the basal activity derived from the native HDAC6 in the cells. We used RNA interference to decrease the amount of endogenous HDAC6. We analyzed HDAC6 immunoreactivity in the cells and confirmed a decrease in HDAC6 expression in cells transfected with a HDAC6 shRNA vector compared with a control shRNA vector (Fig. 7A,C). Hyperacetylation of tubulin was detected in HDAC6-knockdown cells but not in the control cells (Fig. 7B,C).

To examine the effects of the HDAC6 mutants on the level of tubulin acetylation, these mutants and the HDAC6 shRNA vector were transiently co-transfected in SH-SY5Y cells. The expression of exogenous wild-type HDAC6 restored acetylation of tubulin to normal levels (Fig. 7D,E and supplementary material Fig. S3B). Expression of mutant S458A caused the hyperacetylation of tubulin (Fig. 7D,E and supplementary material Fig. S3B). This indicated that the HDAC6 S458A mutant could not compensate for endogenous HDAC6 suppression by transfection with the HDAC6 shRNA vector. However, S458E caused a slight decrease in tubulin acetylation (Fig. 7D,E and supplementary material Fig. S3B). We examined whether rotenone still induced aggresomes in cells expressing the HDAC6 mutant. Wild-type and S458E HDAC6 induced formation of aggresomes of moderate size in the rotenone-treated cells (Fig. 8A). However, S458A formed only aggregates with blurred margins, which were clearly different from typical rotenone-induced aggresomes (Fig. 8B). To examine biochemically the deacetylase activity of HDAC6 mutants, we used the immunoprecipitates of exogenous HDAC6 derived from mutant-expressing cells and measured the deacetylase activity. S458A

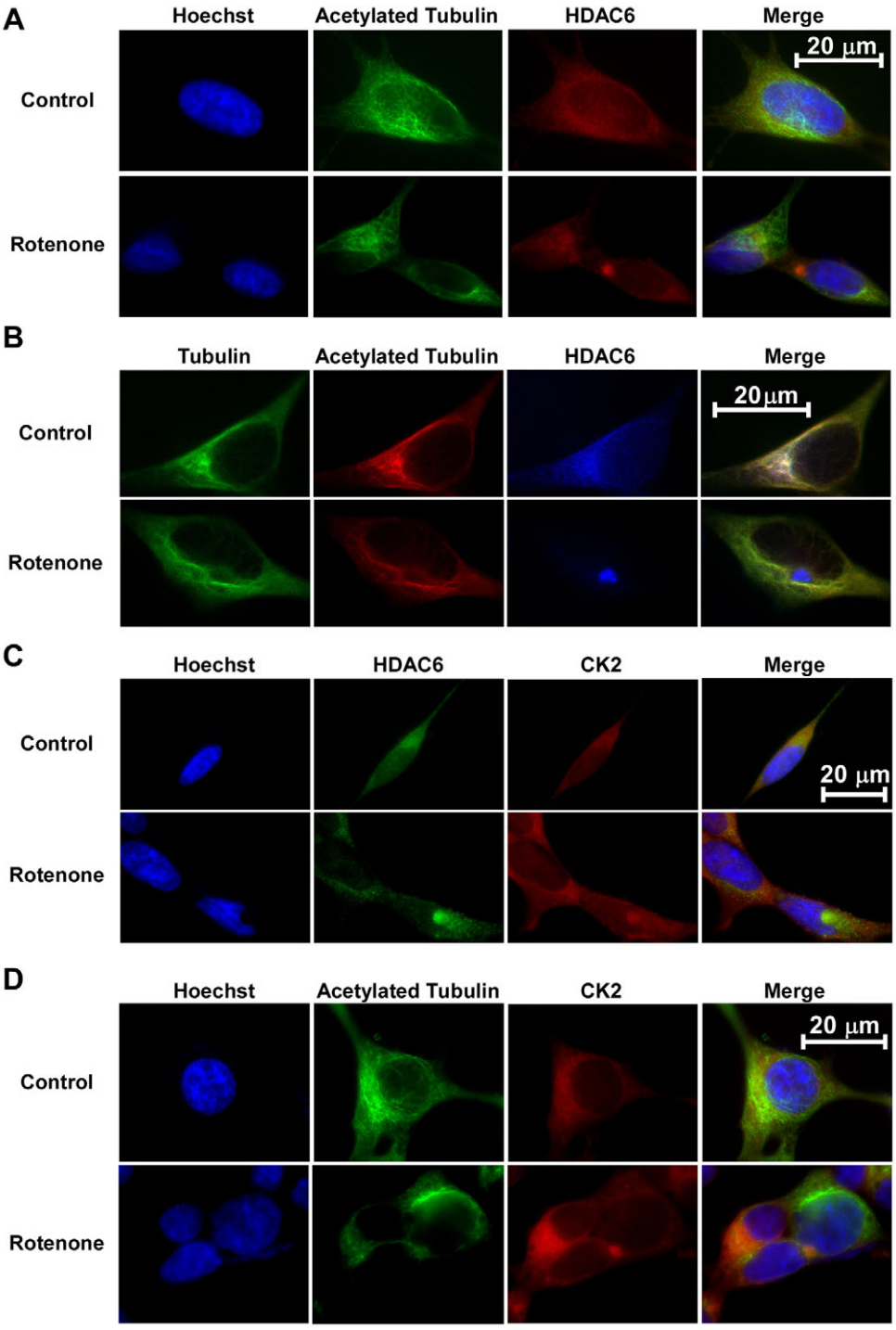

Fig. 5. Detection of aggresome and changes in CK2 tubulin acetylation in rotenone-treated SH-SY5Y cells. SH-SY5Y cells were treated with 0.04 μM rotenone for 72 hours. The nuclei were stained with Hoechst (blue), and aggresome-related proteins were immunocytochemically detected using specific antibodies: (**A**) HDAC6 (red) and acetylated α-tubulin (green), (**B**) HDAC6 (blue), α-tubulin (green) and acetylated α-tubulin (red), (**C**) HDAC6 (green) and CK2α (red), (**D**) acetylated α-tubulin (green) and CK2α (red).

HDAC6 caused a decrease in deacetylase activity compared with the wild-type level, whereas S458E caused a marked increase (Fig. 8C).

HDAC6 phosphorylation by CK2 is associated with the clearance of misfolded protein aggregates and cell viability in response to stress

We examined the relationship between CK2-induced HDAC6 phosphorylation and the clearance of misfolded protein aggregates and whether or not rotenone induced activation of autophagy in our model. We assessed autophagy activation by monitoring the conversion of LC3 from the LC3-I cytosolic form to the LC3-II autophagosome-associated form. Rotenone induced the conversion of LC3-I to LC3-II (Fig. 9A,B), suggesting that rotenone induced the autophagy system for the clearance of aggresomes containing misfolded protein aggregates. However, there was no apparent relationship between HDAC6 and the activation of autophagy (Lee et al., 2010). Aggresomes must be fused with lysosomes for the clearance of misfolded protein aggregates. HDAC6 recruits and deacetylates cortactin, which is a key component of the F-actin polymerization machinery (Zhang et al., 2007), which is essential for aggresome–lysosome fusion and clearance (Lee et al., 2010).

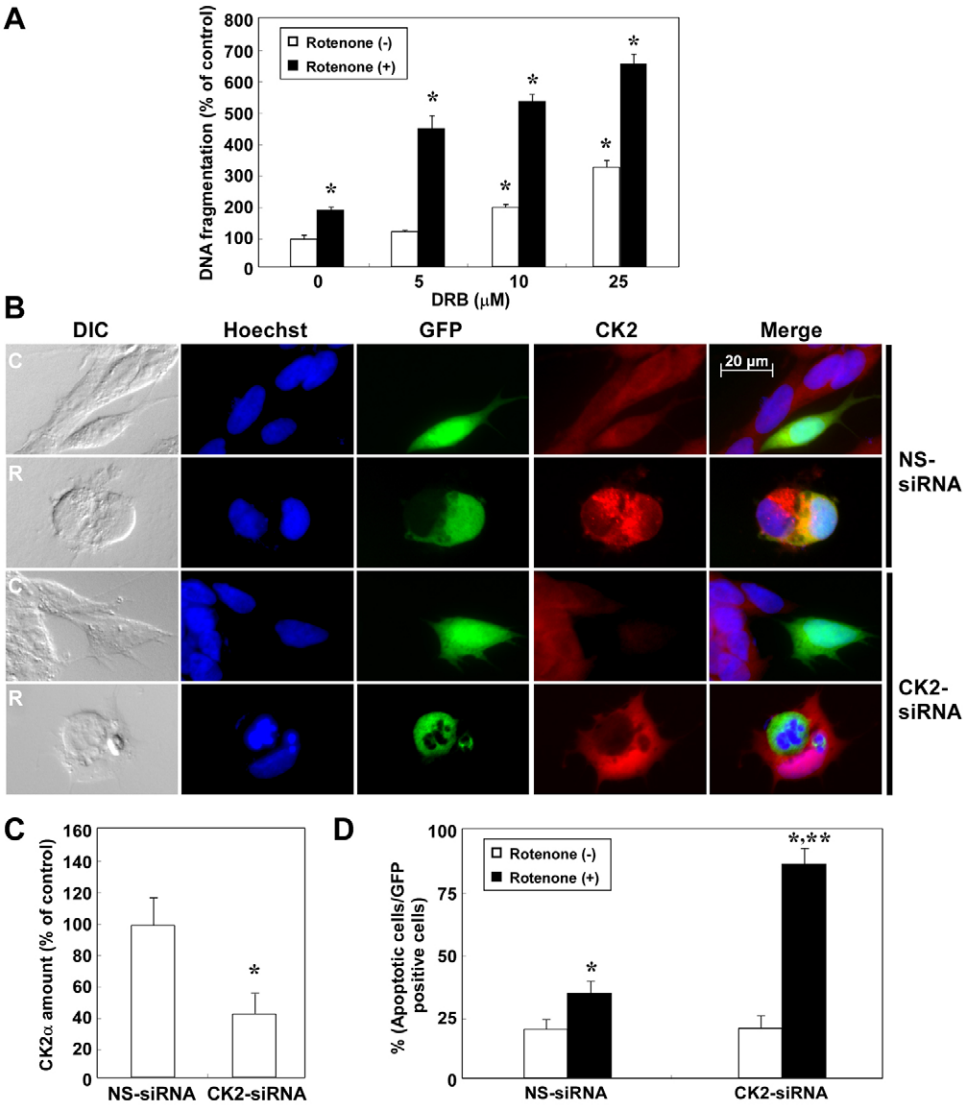

Fig. 6. Effect of CK2 on rotenone-induced apoptosis in SH-SY5Y cells. (**A**) DNA fragmentation in SH-SY5Y cells treated with 0.04 μM rotenone in the presence or absence of DRB at the indicated concentrations for 72 hours. (**B**) After SH-SY5Y cells had been co-transfected with pGFP and non-silencing (NS) siRNA or siRNA against CK2α/α′, they were treated with 0.04 μM rotenone for 48 hours. The intracellular localization of CK2α (red) and the nuclei (blue; Hoechst staining) were detected immunocytochemically. GFP-positive cells (green) were considered to be siRNA-transfected cells. DIC, Nomarski differential interference contrast. (**C**) Fluorescence intensity changes in cells transfected with CK2α/α′ siRNA (GFP-positive cells) quantified in comparison with the intensity of NS siRNA cells. (**D**) Percentage of GFP-positive cells that were apoptotic. C, control; R, rotenone treatment.

We then examined the level of acetylated cortactin. Rotenone induced a decrease in acetylated cortactin (Fig. 9C). DRB, L-NAME and NAC restored the level of acetylated cortactin (Fig. 9C). We examined immunocytochemically the intracellular localization of HDAC6 and cortactin. Rotenone induced colocalization of cortactin to aggresome-like aggregates containing HDAC6 (Fig. 9D). F-actin was also concentrated at cortactin-positive aggregates containing HDAC6 (Fig. 9E).

We next examined whether the rotenone-induced autophagolysosome forms normally in cells expressing mutant HDAC6 by monitoring the colocalization of cortactin to aggresome-like aggregates containing HDAC6. The wild-type and S458E HDAC6 induced colocalization of cortactin to aggresome-like aggregates of moderate size containing HDAC6 in rotenone-treated cells (Fig. 10B). However, S458A did not form distinct aggregates, and cortactin was not concentrated at HDAC6-positive aggregates (Fig. 10B). Furthermore, chromatin condensation in nuclei was also observed (supplementary material Fig. S4B). S458A-expressing cells were vulnerable to rotenone compared with cells expressing wild-type or S458E HDAC6 (Fig. 10C). Interestingly, the expression of S458E HDAC6 slightly induced chromatin condensation in nuclei (supplementary material Fig. S4A) and decreased cell viability, even in the absence of rotenone (Fig. 10C). This phenomenon might be related to the acceleration of tubulin deacetylation, because the acetylation of tubulin is important for cell stability (Matsuyama et al., 2002; Tran et al., 2007; Zilberman et al., 2009). Together, these results demonstrate that CK2 is essential for cell viability in response to misfolded protein stress.

Discussion

CK2 is a regulator of HDAC6 catalytic activity and a bridge between HDAC6 and dynein

We first hypothesized that HDAC6 is a substrate of CK2. According to the consensus phosphorylation site of CK2, (S/T)xx(D/E), human HDAC6 has 26 potential CK2 phosphorylation sites. After confirmation of the binding between HDAC6 and CK2 by pull-down assay, we demonstrated that CK2 phosphorylates HDAC6 at Ser458 (Fig. 1). The phosphorylation of HDAC6 at Ser22 (Beausoleil et al., 2004) and Thr30 (Molina et al., 2007) in HDAC6 has been reported by the large-scale characterization of nuclear phosphoproteins and the global proteomic profiling of phosphopeptides, but the role of phosphorylation in these sites is unknown. The phosphorylation of Tyr570 in deacetylase domain 2 of HDAC6 by EGF receptor results in reduced deacetylase activity

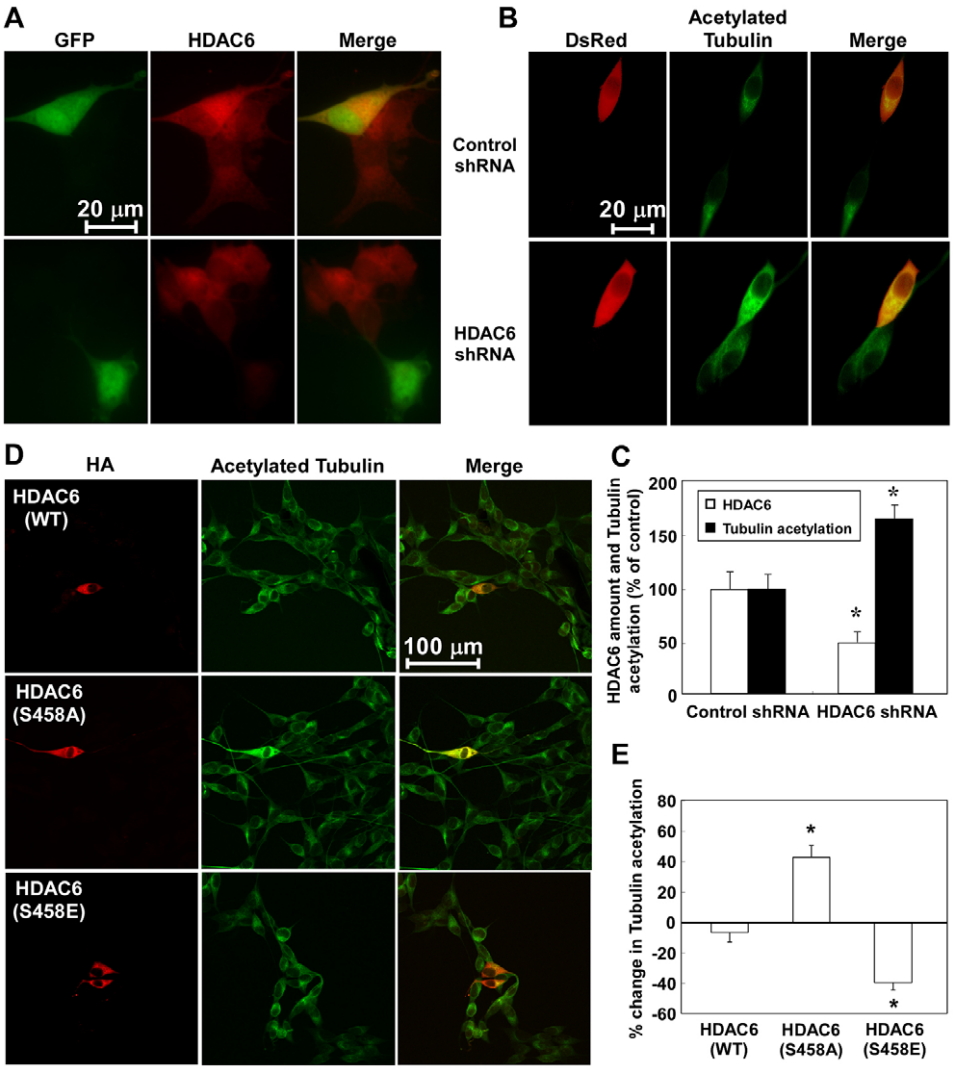

Fig. 7. Effects of HDAC6 shRNA and HDAC6 mutants on tubulin acetylation. (**A**) After SH-SY5Y cells had been co-transfected with pGFP and non-silencing (control) shRNA or HDAC6 shRNA for 72 hours, the intracellular localization of HDAC6 (red) was detected immunocytochemically. GFP-positive cells (green) were considered to be shRNA plasmid-transfected cells. (**B**) After SH-SY5Y cells had been co-transfected with pDsRed and non-silencing (control) shRNA or HDAC6 shRNA for 72 hours, the intracellular acetylated level of tubulin (green) was detected immunocytochemically. DsRed-positive cells (red) were considered to be shRNA plasmid-transfected cells. (**C**) Fluorescence intensity changes in HDAC6 shRNA-transfected cells (GFP- or DsRed-positive cells) were quantified in comparison with the intensity of non-silencing (control) shRNA. (**D**) After SH-SY5Y cells had been co-transfected with HDAC6 shRNA and recombinant HDAC6 mutants [HA-tagged HDAC6 wild type (WT), HA-tagged HDAC6 mutants (S458A and S458E)], cells were cultured for 72 hours. The intracellular acetylated levels of tubulin (green) and recombinant HDAC6s (red; HA) were detected immunocytochemically. HA-positive cells (red) were considered to be shRNA plasmid-transfected cells. (**E**) Fluorescence intensity changes in the cells transfected with HDAC6 shRNA followed by recombinant HDAC6 mutants[HA-tagged HDAC6 wild type (WT), HA-tagged HDAC6 mutants (S458A and S458E)]. Tubulin acetylation (green) in HA-positive cells was quantified in comparison with that of HA-negative cells, the value for which was taken as 0%.

(Deribe et al., 2009). Here, we show that HDAC deacetylase activity is upregulated by the phosphorylation of Ser458.

Because the newly identified phosphorylation site localizes in the dynein motor binding domain (Kawaguchi et al., 2003), it appeared reasonable to consider that CK2 might be involved in the regulation of binding between HDAC6 and dynein. We examined whether the phosphorylation of HDAC6 by CK2 is required for the binding of HDAC6 and dynein. Despite excess amounts of both HDAC6 and dynein, binding of HDAC6 to dynein was slight in the absence of CK2, and this binding was increased by the addition of CK2 (Fig. 2B). These results might indicate that CK2-mediated phosphorylation is required for sufficient binding of HDAC6 and dynein. But, in contrast to our expectations, the catalytic activity of CK2 was not required for the formation of HDAC6 and dynein complexes.

Because CK2 binds to and phosphorylates the dynein motor (Karki et al., 1997), CK2 was found in our immunoprecipitates of endogenous dynein brought down from human SH-SY5Y cells. Kawaguchi and colleagues used similar immunoprecipitates (Kawaguchi et al., 2003) and first reported the binding of HDAC6 and dynein. Indeed, faint bands of CK2 were detected in our immunoprecipitates (Fig. 2B). However, the amount of HDAC6 bound to dynein was slight, despite the excess amounts of HDAC6 and dynein. These findings indicate that the increased amount, but not the catalytic activity, of CK2 is required for the increase in formation of HDAC6 and dynein complexes. CK2 is a microtubule-associated protein that potently induces microtubule assembly and bundling. Similarly to results in the present study, in a previous study the kinase activity of CK2 was not required for its microtubule-assembling and -stabilizing function (Lim et al., 2004b). CK2 is also an inhibitor of the neuronal Cdk5 and blocks the formation of Cdk5 and p35 complexes by the direct binding of CK2, where the kinase function of CK2 is not required for Cdk5 inhibition (Lim et al., 2004a). Thus, CK2 functions as a bridge for binding of HDAC6 and dynein, without a phosphorylation reaction.

CK2 is expressed at low levels in all eukaryotic cells and is a constitutively active enzyme (Filhol and Cochet, 2009). However, it remains unclear, how, when and where the CK2 kinase activity is regulated in cells (Miyata, 2009). Changes in CK2 activity induced by various stimuli are correlated with its protein levels (Takahashi et al., 2007). CK2β is normally expressed at a higher level than the catalytic subunits (α and α′) of CK2, allowing some CK2β to be incorporated into tetramers and stabilized, whereas the excess CK2β is rapidly degraded with a half-life of less than 1 hour (Luscher and Litchfield, 1994). The observed ubiquitylation of CK2β and the accumulation of CK2β protein upon proteasome inhibition suggested

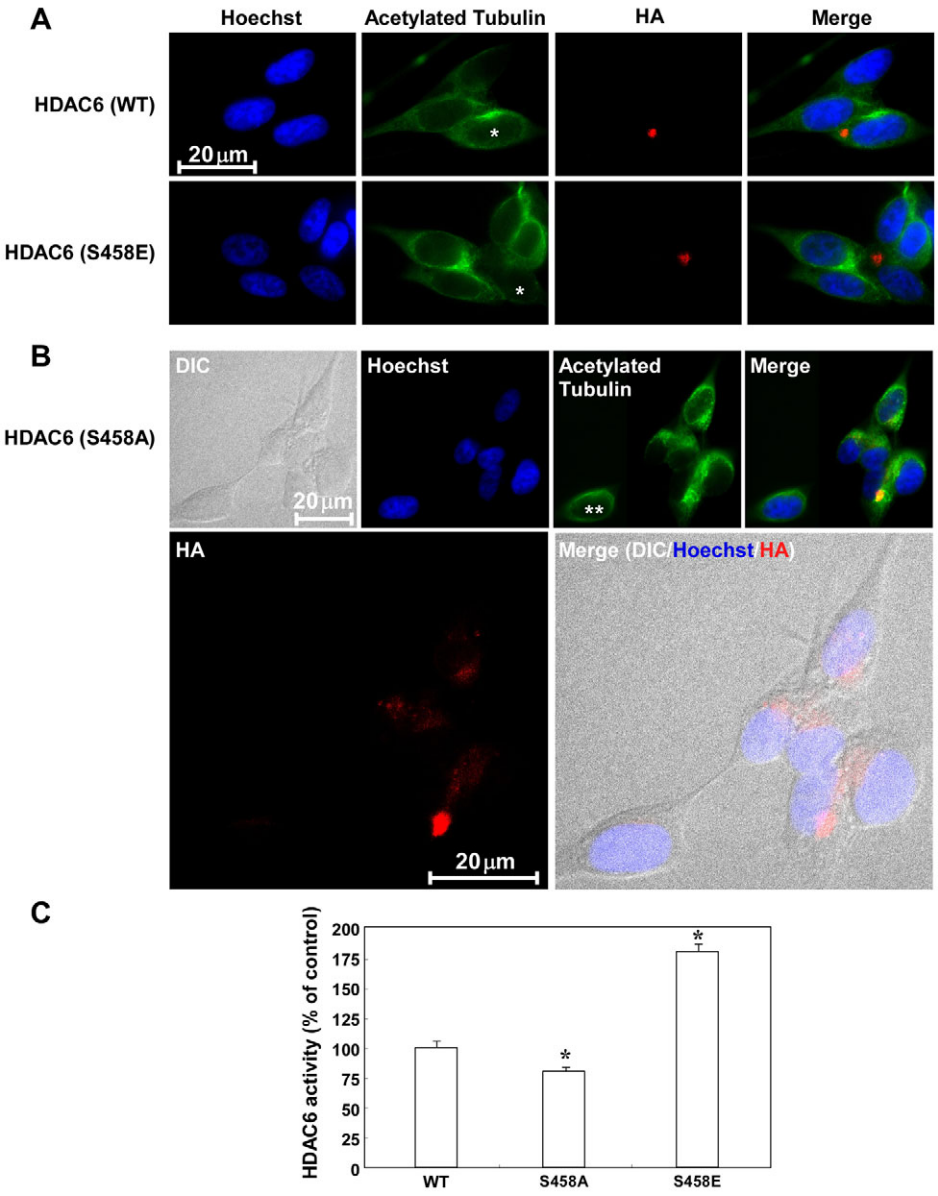

Fig. 8. Effects of HDAC6 shRNA and HDAC6 mutants on aggresome formation. After SH-SY5Y cells had been co-transfected with HDAC6 shRNA and recombinant HDAC6 mutants [HA-tagged HDAC6 wild type (WT), HA-tagged HDAC6 mutants (S458A and S458E)], they were cultured for 72 hours in the presence (A,B) or absence (C) of rotenone. (**A**) Using cells expressing WT and S458E, the intracellular localization of acetylated α-tubulin (green), HDAC6s (red; HA) and the nuclei (blue; Hoechst staining) was detected immunocytochemically. Asterisk indicates transfected cells. (**B**) Using cells expressing S458A, the intracellular localization of acetylated α-tubulin (green), HDAC6s (red; HA) and the nuclei (blue; Hoechst staining) were detected immunocytochemically. Asterisks indicate untransfected cells. (**C**) HDAC6 activity in immunoprecipitates with anti-HA antibody and cell lysates.

that polyubiquitylation of CK2β targets it for degradation (Zhang et al., 2002). Indeed, CK2β localizes in Lewy bodies in Parkinson's disease (Ryu et al., 2008). Because CK2β is thus expressed at a higher level than the catalytic subunit, the expression level of the catalytic subunit determines the CK2 protein kinase activity. Furthermore, by virtue of the importance of subunits, CK2α- or CK2β-knockout mice are lethal (Buchou et al., 2003; Lou et al., 2008), whereas CK2α′-knockout mice are viable (Xu et al., 1999). Therefore, the change in concentration of CK2α is essential for many reactions in response to misfolded protein stress.

Phosphorylation of HDAC6 by CK2 controls aggresome formation and clearance

Rotenone inactivates proteasome by oxidative modification and induces protein aggregates of high molecular weight in SH-SY5Y cells (Shamoto-Nagai et al., 2003). Rotenone induced an increase in concentration of HDAC6 and the dynein complex, which was accompanied by an increase in CK2 binding to HDAC6. Furthermore, rotenone caused phosphorylation at the serine residue of HDAC6, which was suppressed by a CK2-specific inhibitor (Fig. 4C). Thus, phosphorylation at the serine residue of HDAC6 by CK2 occurs under the experimental conditions in which aggresomes are formed. Under the same conditions, HDAC6 activity was increased and the acetylation of tubulin, which is a major substrate of HDAC6, was decreased; both phenomena were suppressed by a CK2-specific inhibitor.

Interestingly, acetylated tubulin was immunocytochemically detected in control cells, even though HDAC6 was also detected in the same area (Fig. 5A,B). This result indicates that the amount of HDAC6 by itself was not sufficient to exert the catalytic activity of HDAC6. We demonstrated that HDAC6 is phosphorylated and activated by CK2 (Fig. 1B; Fig. 2E). p38 is known to activate CK2 (Sayed et al., 2000), and rotenone activates p38 in SH-SY5Y cells (Newhouse et al., 2004). Therefore, it is possible that activation of HDAC6 deacetylase might be caused by the increase in CK2 activity via rotenone-activated p38.

We demonstrated the importance of Ser458 phosphorylation by the use of HDAC6 alanine mutant S458A. S458A mutant failed to

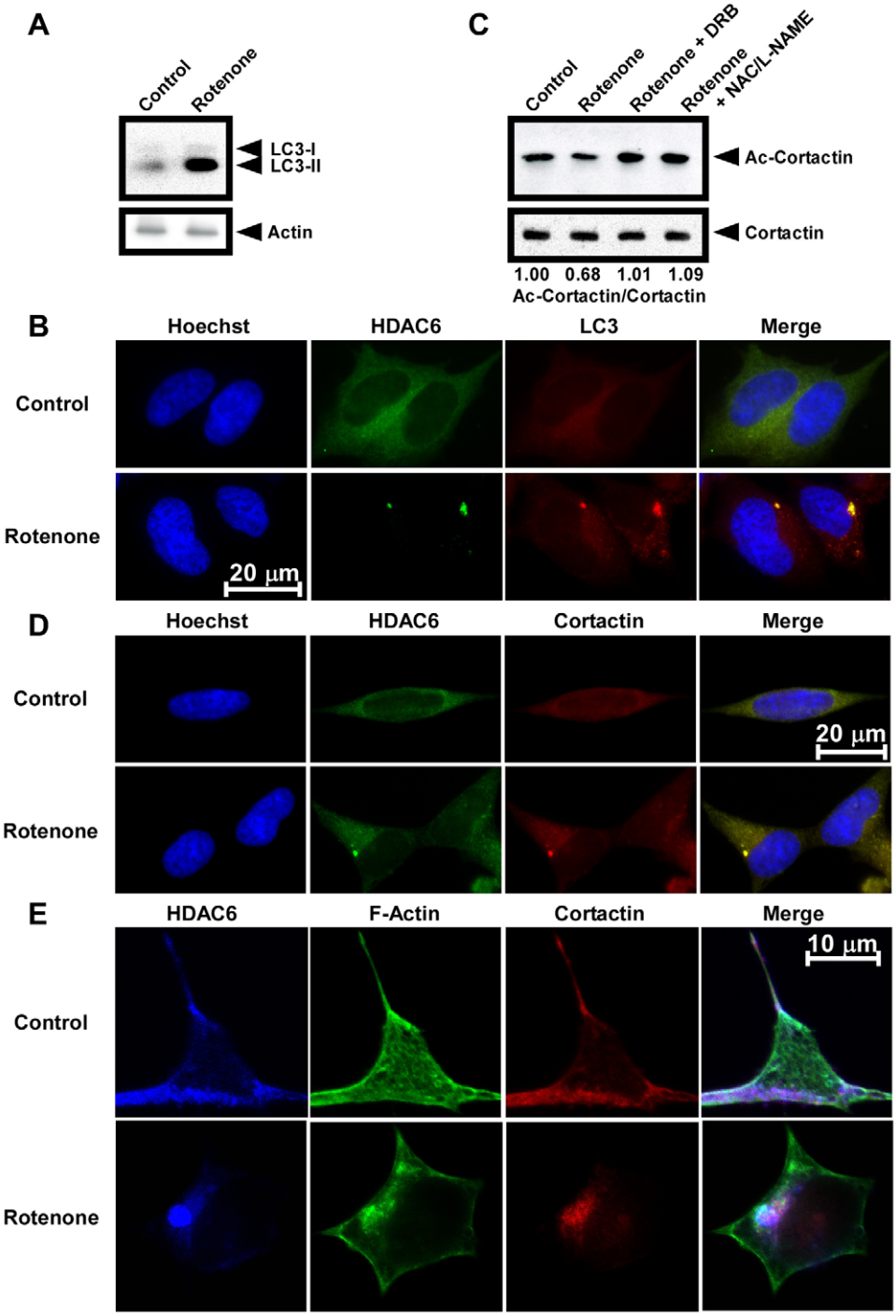

Fig. 9. Effects of rotenone on LC-3 and cortactin in SH-SY5Y cells. SH-SY5Y cells were treated with 0.04 μM rotenone for 72 hours in the presence or absence of 10 μM DRB, 1 mM L-NAME and 3 mM NAC. (**A**) Immunoblot analysis with anti-LC3 and anti-actin antibodies. (**B**) Nuclei were stained with Hoechst (blue) and proteins were immunocytochemically detected using anti-HDAC6 (green) and LC3 (red) antibodies. (**C**) Immunoblot analysis using anti-acetylated cortactin and anti-cortactin antibodies. (**D**,**E**) The nuclei were stained with Hoechst (blue) and proteins were immunocytochemically detected using each specific antibody; (D) HDAC6 (green), cortactin (red), (E) HDAC6 (blue), F-actin (green) and cortactin (red).

functionally recover the decrease of endogenous HDAC6, to recruit misfolded protein aggregates to MTOC or to promote autophagy by recruiting cortactin-dependent, actin-remodeling machinery (Fig. 10B).

However, HDAC6 glutamic acid mutant S458E functionally recovered from the decrease in endogenous HDAC6 by HDAC6 shRNA vector, indicating the weak cytotoxicity. Because HDAC6-mediated tubulin deacetylation destabilizes the dynamic microtubules (Matsuyama et al., 2002), the expression of HDAC6 S458E mutant, which is constitutively active, showed weak cytotoxicity by destabilizing the dynamic microtubules. Indeed, HDAC6 overexpression prompts drug-induced depolymerization (Matsuyama et al., 2002) and the disruption of the microtubule network increases the cytotoxicity (Taylor et al., 2003).

HDAC6 has been reported to bind directly to protein phosphatase 1 (PP1) (Brush et al., 2004). PP1 is an essential Ser/Thr phosphatase found in all tissues with roles in many cellular responses (Cohen, 2002). Its activity is exceedingly tightly and specificly regulated by more than 100 inhibitor proteins (Bollen, 2001; Watanabe et al., 2001). One of these is a dopamine- and cAMP-regulated phosphoprotein, DARPP32, which requires phosphorylation at Thr34 by protein kinase A (PKA) to become active as a PP1

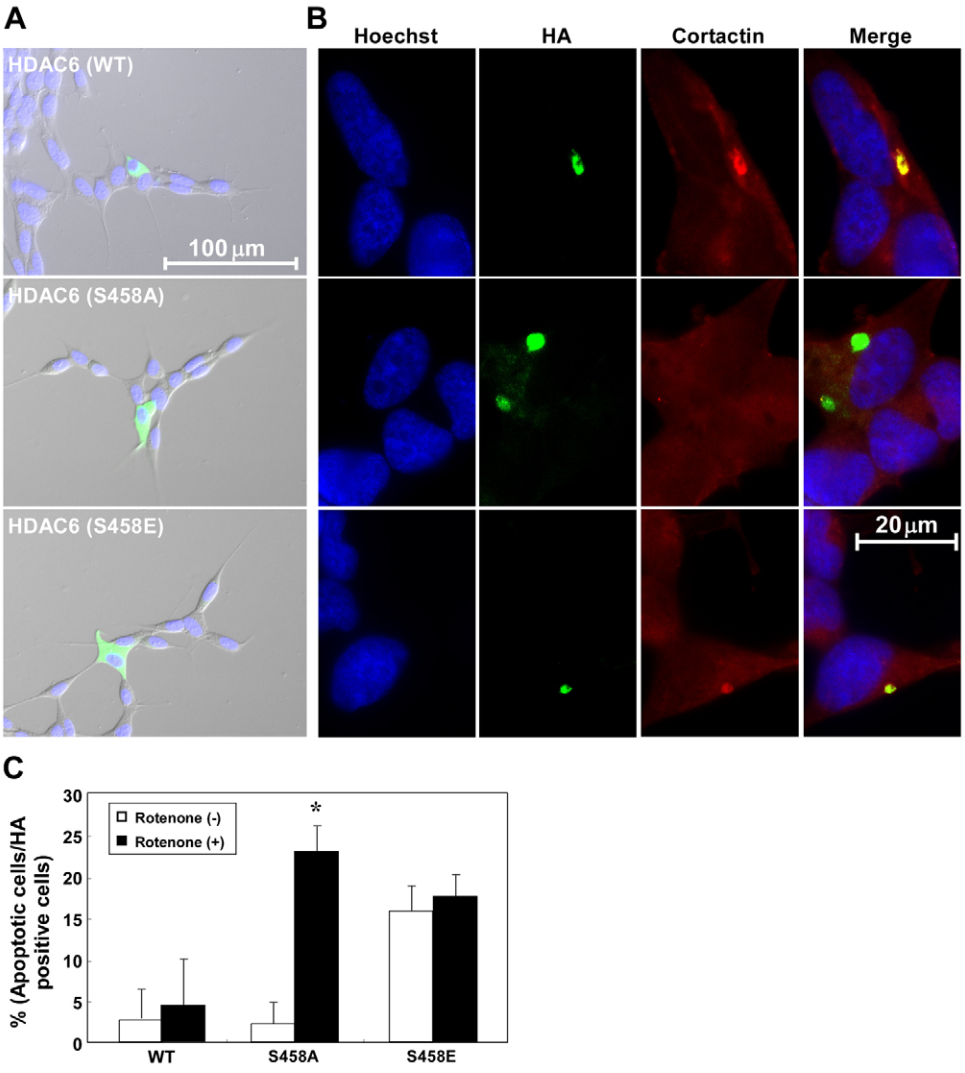

Fig. 10. Effects of HDAC6 mutants on HDAC6-recruited cortactin and apoptosis induced by rotenone. After SH-SY5Y cells had been co-transfected with HDAC6 shRNA and various HDAC6-expressed vectors [HA-tagged HDAC6 wild type (WT) and HA-tagged HDAC6 mutants (S458A and S458E)], they were cultured for 72 hours in the absence (**A**) or presence (**B**,**C**) of rotenone. The nuclei were stained with Hoechst (blue), and proteins were stained with anti-HA (green) and cortactin (red) antibodies (A,B). (C) Percentage of HA-positive cells undergoing apoptosis.

inhibitor (Hemmings et al., 1984). As Gao and co-workers previously reported, a portion of rotenone signals are mediated via the PKA pathway (Gao et al., 2008), and rotenone might cause the inhibition of PP1 through DARPP32 phosphorylation by PKA in the process of aggresome formation. Therefore, the quantitative balance between CK2 and PP1 bound to HDAC6 might determine the level of phosphorylation.

These results indicate that CK2 phosphorylates HDAC6 at Ser458 to induce deacetylation of tubulin and the recruitment of misfolded protein aggregates to MTOC by motor proteins.

The regulatory mechanism against misfolded protein stress response is conserved only in higher primates

The identified Ser458 as the CK2-mediated phosphorylation site of HDAC6 is conserved only in higher primates such as human and chimpanzee, and not in mouse, rat, dog, bovine or rhesus macaque. This information might be very interesting in terms of the evolutional process. Because this phosphorylation has an important role in the activation of HDAC6 deacetylase activity, we conclude that this regulatory mechanism by phosphorylation exists only in higher primates.

In summary, our results demonstrate that the CK2-mediated phosphorylation of HDAC6 regulates the recruitment of misfolded proteins to the MTOC for aggresome formation. This involves the dynein motor proteins and also regulates cortactin-recruited actin-remodeling machinery, leading to autophagy. The clearance of misfolded protein aggregates from many normal cellular processes is crucial for cell viability, and abnormality of this regulation might result in many pathological conditions, including neurodegenerative diseases. Furthermore, because the newly identified CK2-mediated phosphorylation site of HDAC6 is conserved only in higher primates, such as human and chimpanzee, our findings might also have an evolutionary significance.

Materials and Methods

Materials

Rotenone, N-acetyl cysteine (NAC) and N-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma. GST–HDAC6 was from BPS Bioscience and human recombinant CK2β was from ATGen. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), 4,5,6,7-tetrabromobenzotriazole (TBB), human recombinant CK2 and human recombinant CK2α were from Calbiochem. Alexa-Fluor-488–phalloidin was from Invitrogen.

Antibodies

Anti-β-tubulin, γ-tubulin, acetylated-α-tubulin, vimentin, dynein, phosphorylated-Ser and actin antibodies were purchased from Sigma. Antibodies against HDAC6, α-tubulin, CK2 β and ubiquitin were from Santa Cruz Biotechnology, anti-phospho (Ser129) α-synuclein antibody and anti-HA were from Wako Pure Chemical Industries; casein kinase 2α (CK2α), parkin (PRK8), nitrotyrosine, cortactin and acetylated-cortactin antibodies were from Millipore Bioscience Research Reagents.

Anti-LC3B (D11) XP antibody was from Cell Signaling Technology, and anti-HRP-conjugated GST-Tag antibody was from MBL International.

Pull-down assay

GSH-Sepharose beads pre-bound with GST-HDAC6 were incubated with various recombinant CK2 proteins for 1 hour at 30°C. After being extensively washed with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM $MgCl_2$), the beads were boiled in SDS-PAGE sample buffer and analyzed by immunoblotting.

Immunoblot analysis

Immunoblotting was performed as described previously (Watabe et al., 1998). Briefly, cells were lysed in SDS-PAGE sample buffer and the cell lysate was then boiled. Denatured proteins were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (GE Healthcare). The membrane was incubated with a blocking solution [2% bovine serum albumin (Sigma) dissolved in phosphate-buffered saline (PBS) containing 0.2% Tween 20] for 1 hour at room temperature, washed with PBS containing 0.2% Tween 20 and incubated with the first antibody dissolved in the blocking solution overnight at 4°C. After washing, the membrane was incubated for 1 hour with horseradish-linked secondary antibody. Immunoreactive proteins were detected with an enhanced chemiluminescence system (GE Healthcare).

In vitro kinase assay

An in vitro kinase assay was performed as described previously (Watabe et al., 1997). GST–HDAC6 and CK2 were added to a kinase buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM $MgCl_2$). The reaction was started by adding a 0.1 mM ATP containing [γ-^{32}P]ATP and incubation for 30 minutes at 30°C. After SDS-PAGE, the incorporation of ^{32}P into the proteins was evaluated with the image analyzer FLA-7000 (Fujifilm).

Phosphoamino acid analysis

After SDS-PAGE and electrophoretic transfer to a polyvinylidene difluoride membrane (Bio-Rad), radioactively labeled, phosphorylated HDAC6 was identified by autoradiography, cut and subjected to acid hydrolysis with 6 N HCl for 16 hours at 110°C. Phosphoamino acids in the hydrolysate were separated by thin-layer chromatography on cellulose plates. Positions of phosphoamino acid standards (2 μg/μl each of phosphoserine, phosphothreonine, and phosphotyrosine) were visualized by ninhydrin staining, and ^{32}P-labeled phosphoamino acids were detected by autoradiography.

Phosphorylation of peptide array

An array of 18 peptides was synthesized on cellulose paper as a PepSpot (JPT Peptide Technologies). PepSpot was moistened in methanol and incubated for 2 hours at room temperature in a kinase buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM $MgCl_2$) supplemented with 1 mg/ml bovine serum albumin but lacking ATP. The solution was decanted, the kinase buffer containing 10 μM ATP was added and the reaction vessel was equilibrated for 30 minutes at 30°C. After this blocking step, the buffer was changed for the kinase buffer containing [γ-^{32}P]ATP. The reaction was started by adding a human recombinant CK2, and PepSpot was incubated for 30 minutes at 30°C with slight agitation. After extensive washing with phosphate, PepSpot was air-dried and the incorporation of ^{32}P into the peptides was evaluated with the image analyzer FLA-7000 (Fujifilm, Japan).

Identification of CK2 phosphorylation site in HDAC6

To determine the phosphorylation site, HDAC6 was phosphorylated by CK2 and purified by SDS-PAGE. The gel band corresponding to the protein was excised, and the protein was digested using a Trypsin Profile IGD Kit (Sigma). The digested protein was then analyzed by MALDI mass spectrometry using a MALDI-TOF AXIMA Performance (Shimadzu Biotech) and α-cyano-4-hydroxycinnamic acid as matrix (Sigma). The protein fingerprinting was analyzed with Mascot search engine (Matrix Science) to highlight possible phosphorylated peptides, using Ser/Thr phosphorylation as a variable modification. A peptide corresponding to the sequence (448–480) of HDAC6 was identified as possibly phosphorylated. The exact phosphorylated site was identified by acquiring an MS/MS spectrum, and the position of the phosphorylated site was determined.

HDAC6 activity

HDAC6 activity was measured using an HDAC Fluorimetric Assay kit (Biomol) according to the manufacturer's instructions.

Cell culture

SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C under 5% CO_2 in air.

Immunofluorescence microscopy

Immunocytochemical study was performed as described previously (Watabe et al., 2000). Cells were washed with PBS and fixed with 3.7% formaldehyde for 20 minutes. Cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 minutes and then washed three times with PBS. Incubation with primary antibody was carried out for 1 hour at room temperature. Excess antibody was washed out three times with PBS. This was followed by incubation with an appropriate fluorophore-labeled secondary antibody for 1 hour at room temperature in an area protected from light. After washing out the excess antibody three times with PBS, mounting was performed using a ProLong Antifade Kit (Molecular Probes). Hoechst 33342 staining was performed as described (Watabe and Nakaki, 2004). Images were obtained by an Axio Imager M1 fluorescence microscope using Axio Vision software (Carl Zeiss) and an FV1000-MPE laser-scanning fluorescent microscope using FV1000-D software (Olympus).

Immunoprecipitation

Immunoprecipitation was performed using a method modified from that described previously (Watabe et al., 1997). After treatment with rotenone, the cells were pelleted, washed twice in PBS and lysed in lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) containing a protease inhibitor cocktail (Sigma). The cell lysates were then used for immunoprecipitation in the presence of a specific antibody. Immune complexes were precipitated using Protein G PLUS/Protein A-Agarose (Calbiochem) and washed four times in lysis buffer. The precipitate was resuspended in SDS-PAGE sample buffer and boiled. Immunoblot analysis was then performed.

Quantification of DNA fragmentation

As described previously (Watabe et al., 2004), DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Synthesis of siRNA

Chemically synthesized siRNAs were obtained from Sigma. The siRNA sequence of human CK2α/α′ was used as previously reported (Lim et al., 2004b). A non-silencing RNA duplex was used as a control (Kim et al., 2005). Non-target shRNA control vector and HDAC6 shRNA vector were obtained from Sigma.

Plasmids and mutagenesis

An expression vector containing a human *HDAC6* cDNA, pReceiver-M45-HDAC6, was purchased from GeneCopoeia. The S458A and S458E single mutant cDNAs were obtained by site-directed mutagenesis using a PCR overlap extension method (BEX). These cDNAs were analyzed on both strands by DNA sequencing to ensure there were no unwanted changes in the codons. The vectors containing wild-type and mutant *HDAC6* cDNAs allowed the expression of C-terminally HA-tagged wild-type and mutant HDAC6 proteins in mammalian cells. All expression constructs were sequenced to confirm that the fusion was in the correct reading frame.

Transfection

Cells were transfected with siRNAs or plasmids using Lipofectamine RNAiMAX (Invitrogen) or NeuroFect^{TM} (Genlantis), as described previously (Watabe and Nakaki, 2004).

Statistical analysis

Statistical significance was determined using either Student's *t*-test for two-group comparisons or ANOVA with the Mann-Whitney *U*-test for multiple group comparisons. $P<0.05$ was considered significant.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/9/1519/DC1

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