Cell death triggered by polyglutamine-expanded huntingtin in a neuronal cell line is associated with degradation of CREB-binding protein

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Received July 10, 2002; Revised October 10, 2002; Accepted October 30, 2002

Huntington’s Disease belongs to the CAG repeat family of neurodegenerative diseases and is characterized by the presence of an expanded polyglutamine (polyQ) repeat in the huntingtin (htt) gene product. PolyQ-expanded htt accumulates within large aggregates that are found in various subcellular compartments, but are more often localized within the nucleus. It has been suggested that the sequestration of proteins essential to cell viability may be one mechanism that accounts for toxicity generated by polyQ-expanded proteins. Nuclear inclusions containing polyQ-expanded htt recruit the transcriptional cofactor, CREB-binding protein (CBP). PolyQ toxicity appears to involve alterations of gene transcription and reduced neuronal cell viability. In the HT22 hippocampal cell line, we find that toxicity within individual cells induced by polyQ-expanded htt, as revealed by a TUNEL assay, is associated with the localization of the mutant htt within either nuclear or perinuclear aggregates. However, in addition to CBP recruitment, we show here that CBP ubiquitylation and degradation can be selectively enhanced by polyQ-expanded htt. Thus, selected substrates may be directed to the ubiquitin/proteasome-dependent protein degradation pathway in response to polyQ-expanded htt within the nucleus.

INTRODUCTION

Huntington’s Disease (HD) belongs to a family of neurodegenerative diseases that is characterized by the presence of an expanded polyglutamine (polyQ) repeat in the gene product causally linked to disease (1,2). Each of the polyQ-repeat diseases has a characteristic phenotype and neuropathology that is not strictly related to the expression profile of the mutant protein (3). For example, polyQ-expanded huntingtin (htt), the product of the IT15 gene, is expressed in various tissues and brain regions yet degeneration is primarily restricted to striatal and cortical neurons (4).

Degenerating neurons in polyQ repeat diseases accumulate intranuclear inclusions (NIs) that contain aggregated polyQ protein (5). Despite the predominance of NIs, aggregated polyQ protein can also be found in other subcellular compartments in tissues from both human postmortem samples and transgenic mice (6–9). For example, in a transgenic mouse model of spinocerebellar ataxia-2 (SCA-2) or postmortem SCA-2 brain, cytoplasmic aggregates were detected that contained polyQ-expanded ataxin-2 protein (8). Likewise, in HD postmortem brains, aggregates containing polyQ-expanded htt were found in the nucleus, perikarya and processes of the neurons in affected areas (6,7). PolyQ repeat disease proteins also form aggregates that localize in various subcellular compartments when expressed in transfected cell lines of neuronal or nonneuronal origin (10–14).

NIs containing expanded polyQ proteins were thought to contribute to neuronal damage and degeneration in polyQ repeat diseases (5,15,16). However over the past few years, results obtained in a number of in vitro and in vivo models of polyQ repeat diseases have led to a re-examination of the relationship between polyQ-containing NIs and neurodegeneration (17–21). In transfected primary striatal neurons, accumulation of polyQ-expanded htt within nuclear aggregates was enhanced by agents such as BDNF and CTNF, or expression of BCLxl, yet neuronal toxicity was reduced (17). Furthermore, cotransfection of a dominant-negative ubiquitin-conjugating enzyme with polyQ-expanded htt reduced aggregation but led to increased toxicity (17). Finally, in a transgenic mouse model of SCA-1, mutation of the self-association domain of polyQ-expanded ataxin-1 blocked its aggregation, but did not affect its ability to cause degeneration of cerebellar purkinje cells (18). These studies suggest that the formation of...
NIs might initially be beneficial for cell survival and serve to sequester aggregated polyQ-expanded proteins (22–25). This conclusion was supported by results obtained in transgenic mice with a deficiency in the E6-AP Ubiquitin Ligase that expressed polyQ-expanded ataxin-1. In this case, fewer NIs containing the polyQ-expanded ataxin-1 were observed while toxicity in cerebellar Purkinje cells was enhanced (21).

Because many transcription factors or cofactors also contain short polyglutamine repeats or polyglutamine and proline-rich regions (26), several studies have examined whether their association with polyQ repeat proteins can impact gene transcription. p53, TAFII130 and CREB-binding protein (CBP) have been found to interact with polyQ repeat proteins and, as a result, have their transcriptional regulatory activity reduced (27–30). Effects of expanded polyQ repeat proteins on CBP, a transcriptional coactivator, have received considerable attention, given the established role for CBP and its target transcription factors (e.g. CREB) in neuronal cell survival (31,32). CBP is recruited into the polyQ aggregates in cultured cells, primary neurons, transgenic mice and postmortem brains of HD, DRPLA, SBMA and SCA3 patients (29,33). As a result of its association with aggregated expanded polyQ protein, CBP effects on CREB are impaired, leading to reductions in cAMP-induced transcription (29). The importance of this reduction in CBP activity in expanded polyQ toxicity was revealed by experiments that showed a rescue of toxicity in cultured cells and primary neurons expressing polyQ-expanded htt upon CBP overexpression (29).

In this study, we used a hippocampal neuronal cell line (i.e. HT22 cells) to examine the effects of polyQ-expanded htt on CBP localization and expression. As observed in other cell culture systems, the subcellular localization of expanded polyQ htt aggregates was an important determinant of effects on HT22 cell toxicity. While these cells were useful in confirming the recruitment of endogenous CBP into expanded-polyQ htt aggregates, they also revealed unique effects of htt on CBP accumulation. Loss of CBP function associated with polyQ-expanded htt resulted from either CBP recruitment into NIs or its degradation, and was shown to be directly related to toxicity as assessed in single cells by TUNEL assays. Furthermore, the selective enhancement of CBP ubiquitylation in HT22 cells expressing polyQ-expanded htt demonstrates that the ubiquitin/proteasome-dependent protein degradation pathway (UPP) may be mobilized to specific substrates when cells are challenged with disease-causing polyQ-expanded proteins.

RESULTS

Distinct responses of CBP to polyQ-expanded htt in HT22 cells

The mouse HT22 hippocampal neuronal cell line has provided a useful model of neuronal cell death and is particularly sensitive to oxidative stress (34–36). Since oxidative stress may contribute to the demise of vulnerable neurons in HD (37), we used HT22 cells for studies of toxicity upon expression of transfected polyQ expanded htt. HT22 cells were therefore transfected with truncated htt protein that contains 63 N-terminal amino acids and either 99 (Htt-N63-99Q) or 19 (Htt-N63-19Q) consecutive glutamine residues. Htt-N63-99Q, but not Htt-N63-19Q, protein expressed in a variety of
transfected neuronal and non-neuronal cell lines forms aggregates and induces toxicity (10). As will be shown below, this dependence on polyQ repeat length for aggregate formation and toxicity (10,16) is also observed in HT22 cells.

When Htt-N63-99Q protein was visualized in transfected HT22 cells by indirect immunofluorescence (IIF) four distinct staining patterns were observed. In the majority of HT22 cells (typically 35–40%) Htt-N63-99Q was found in large nuclear aggregates, while cytoplasmic aggregates, diffuse cytoplasmic staining and large perinuclear aggregates were also observed. Perinuclear aggregates have been observed previously in 293 cells expressing mutant htt protein (38) and are defined by their localization, size (i.e. larger than nucleoli), and their association with distorted nuclear morphology (Fig. 2H). These diverse staining patterns of a polyQ-expanded protein are not unique to HT22 cells and have been observed in many other transfected cell lines (10,16,39).

Various transcription factors and cofactors are recruited into aggregates containing polyQ-expanded protein (27–29,33). Recruitment of the CBP transcriptional coactivator into polyQ repeat protein aggregates appears to play an important role in in vitro toxicity and is a characteristic of vulnerable brain tissue in HD and mouse models of the disease (29). In HT22 cells, endogenous CBP is recruited into nuclear aggregates containing Htt-N63-99Q protein (Fig. 1A–D). Endogenous CBP is also recruited into perinuclear Htt-N63-99Q protein aggregates (Fig. 2E–H). In cells expressing aggregated cytoplasmic Htt-N63-99Q protein, endogenous CBP exhibits a uniform diffuse nuclear staining pattern (Fig. 2A–D). This apparently normal nuclear staining pattern of CBP is observed in all cells expressing cytoplasmic Htt-N63-99Q protein that is not visibly aggregated (data not shown). In non-transfected HT22 cells or transfected cells expressing Htt-N63-19Q, CBP also shows diffuse nuclear staining (data not shown). Surprisingly, in addition to the recruitment of endogenous CBP into expanded polyQ htt protein aggregates, we noticed many cells where CBP staining was diminished (Fig. 1E–H). As summarized in Figure 3, in ~55% of HT22 cells expressing nuclear aggregates of Htt-N63-99Q, CBP staining was undetected. Endogenous CBP staining was also dramatically reduced in ~40% of transfected HT22 cells containing perinuclear Htt-N63-99Q protein aggregates. Under our IIF conditions, CBP was detected in 98% of HT22 cells expressing diffuse cytoplasmic Htt-N63-99Q protein (Fig. 3), non-transfected cells or those expressing Htt-N63-19Q (data not shown). The apparent loss of endogenous CBP was also observed in ~30% of transiently transfected N2a neuroblastoma cells expressing Htt-N63-99Q (data not shown) and thus is not a property unique to HT22 cells.

Despite the variability in both Htt-N63-99Q localization and the response of CBP (Fig. 3), we were able to detect a reduction in transfected CBP levels by western blot analysis of HT22 cell populations expressing Htt-N63-99Q but not Htt-N63-19Q (Fig. 4). Although reduced CBP accumulation and/or its reduced solubility can account for reductions of CBP levels revealed by western blot analysis, these data support the IIF analysis, where selective CBP loss could be detected within individual cells.

In order to test the selectivity of Htt-N63-99Q-induced effects on CBP, we examined the localization of endogenous SP1
transcription factor in transfected HT22 cells. Previous studies in N2a cells showed that the diffuse nuclear localization of SP1 is maintained in transfected cells expressing polyQ expanded htt protein (29). As shown in Figure 5A–H, the diffuse nuclear staining pattern of SP1 in HT22 cells is not altered upon expression of Htt-N63-99Q protein, irrespective of the localization of htt protein. We also examined the localization of ubiquitin and the HDJ2 molecular chaperone in Htt-N63-99Q transfected HT22 cells. As expected from previous studies in other cell lines and tissues (14,39–41), both ubiquitin (Figs. 6A–H) and HDJ2 (data not shown) were recruited into Htt-N63-99Q nuclear aggregates. Thus, the apparent loss of CBP that results from expression of polyQ-expanded htt within nuclear or perinuclear aggregates is selective and not characteristic of either another htt-interacting transcription factor (i.e. SP1) (42), or other proteins recruited into polyQ-expanded htt aggregates.

![Figure 3. Quantitative analysis of endogenous CBP expression in HT22 cells containing Htt-N63-99Q aggregates. Data shown are average of four independent experiments. Approximately 100 cells containing Htt-N63-99Q aggregates were counted in each experiment. In 95% of HT22 cells containing Htt-N63-99Q nuclear aggregates, CBP is no longer homogeneously distributed throughout the nucleus but either recruited into aggregates (40%) or no longer visible (55%). In contrast, CBP maintains its normal diffuse nuclear staining in cells containing cytoplasmic Htt-N63-99Q aggregates.](image)

![Figure 4. Reduction of soluble CBP levels in HT22 cells expressing Htt-N63-99Q. Soluble protein extracts were prepared from HT22 cells transfected with a CBP expression plasmid, Htt-N63-99Q or Htt-N63-19Q expression plasmids, and then subjected to western blot analysis to detect CBP (top panel). Stripped blots were probed with an anti-lamin B (bottom panel) antibody to provide an internal control for protein loading. Blot shown is representative of three separate transfection experiments where reductions in CBP levels (normalized to lamin B expression) in Htt-N63-99Q expressing cells, relative to those expressing Htt-N63-19Q, varied from 40 to 60%.](image)

![Figure 5. Localization of endogenous SP1 in HT22 cells containing Htt-N63-99Q nuclear or cytoplasmic aggregates. Transfected HT22 cells were co-stained with a rabbit anti-c-myc antibody to reveal Htt-N63-99Q protein in red (A, E) and a mouse anti-SP1 antibody to reveal endogenous SP1 in green (B, F). Areas of overlap between SP1 and Htt-N63-99Q are shown in yellow (C, G). Htt-N63-99Q overlap with nuclei stained blue by DAPI is shown in D and H. Panels A–D show that endogenous SP1 maintains its normal diffuse nuclear staining pattern in cells containing Htt-N63-99Q nuclear aggregates. Panels E–H show that endogenous SP1 maintains its normal diffuse nuclear staining in cells containing cytoplasmic Htt-N63-99Q aggregates.](image)
PolyQ-expanded htt selectively enhances CBP engagement with the UPP

The loss of CBP staining in a subset of cells expressing Htt-N63-99Q could result from either enhanced CBP degradation or its reduced synthesis. Since CBP may be a substrate for proteasome-mediated degradation (43), we examined whether proteasome inhibition would reduce CBP loss observed in some HT22 cells expressing nuclear Htt-N63-99Q. Effects of proteasome inhibitors on the loss of CBP in cells expressing polyQ-expanded htt might also rule out CBP synthesis as a target of mutant htt. As shown in Figure 7 and quantified in Figure 8A, treatment of HT22 cells with the irreversible proteasome inhibitor, lactacystin, significantly reduced the fraction of cells that exhibited reduced CBP expression. In this particular experiment, 40% of cells expressing nuclear aggregates of Htt-N63-99Q exhibited CBP loss in the absence of lactacystin treatment (Fig. 8A). Since the percentage of cells with nuclear aggregates of Htt-N63-99Q was not affected by lactacystin, the decrease in cells showing CBP loss is accompanied by an increase in cells exhibiting diffuse nuclear staining of CBP (Fig. 8A). These results are compatible with previous studies (38) showing lactacystin effects on polyQ-expanded htt aggregation, since lactacystin treatment did affect Htt-N63-99Q nuclear aggregation, leading to an increase in the fraction of cells containing multiple (i.e. >3) nuclear aggregates (Fig. 8B). This increase in Htt-N63-99Q aggregation by lactacystin treatment was also revealed by western blot analysis, which also showed a corresponding reduction in soluble Htt-N63-99Q (Fig. 9). The effectiveness of lactacystin in altering proteasome function was also confirmed by the induction of heat shock protein 70 (hsp70) in lactacystin-treated HT22 cells (data not shown). Hsp70 induction is a hallmark of proteasome inhibition in a number of cell types (44).

The recovery of CBP expression upon lactacystin treatment of HT22 cells expressing nuclear Htt-N63-99Q suggests that CBP recruitment to the UPP may be enhanced as a consequence of polyQ-expanded htt expression. Since proteasome targeting requires ubiquitin modification, we examined whether increased CBP ubiquitylation accompanied Htt-N63-99Q expression. Endogenous CBP ubiquitylation was assessed using immunoprecipitation from extracts prepared following co-transfection of HT22 cells with HA-tagged ubiquitin and Htt-N63 plasmids. Gels used for this western blot analysis were loaded to attain equivalent amounts of total CBP levels/lane (Fig. 10A, top panel) in order to aid in comparison of CBP ubiquitylation. As shown in Figure 10A (bottom panel), endogenous CBP ubiquitylation was enhanced in HT22 cells expressing Htt-N63-99Q, but not Htt-N63-19Q protein. Importantly, detection of ubiquitylated CBP in Htt-N63-99Q transfected cells did not require the pharmacological inhibition of proteasome function, which on its own leads to increased accumulation of ubiquitylated CBP (Fig. 10A, bottom panel).

In order to reveal whether Htt-N63-99Q effects on CBP were selective, we examined glucocorticoid receptor (GR) ubiquitylation in the same extracts used for analysis of CBP ubiquitylation. GR is a substrate for the UPP in HT22 cells, as revealed by the increased accumulation of ubiquitylated receptor upon lactacystin treatment (Fig. 10B). This corroborates previously published work from our laboratory where
proteasome inhibition by MG132 treatment of HT22 cells was found to increase GR ubiquitylation (45). However, unlike CBP, GR ubiquitylation is not affected by Htt-N63-99Q (Fig. 10B). Thus, the enhancement of CBP ubiquitylation by Htt-N63-99Q is selective and does not represent an overall increase in ubiquitin conjugation or proteasome inhibition.

**HT22 cell toxicity is associated with the formation of nuclear aggregates containing polyQ-expanded htt**

The loss of CBP function has been found to be a major factor in the cell death induced by polyQ expanded proteins (28,29,33,46). In HT22 cells, the subcellular localization of Htt-N63-99Q has an impact on CBP recruitment or degradation (see above). In order to examine whether CBP localization has an impact on Htt-N63-99Q-induced toxicity, we used a TUNEL assay to stain transfected HT22 cells. As show in Figure 11 and summarized in Figure 12, 80% of transfected HT22 cells expressing Htt-N63-99Q nuclear aggregates were TUNEL-positive, while only 20% of the cells containing cytoplasmic aggregates of Htt-N63-99Q were TUNEL-positive. These results are consistent with other published studies examining polyQ-expanded htt in transfected cells where the extent of cellular toxicity was correlated with nuclear localization of polyQ-expanded htt (15,47). However, our results are unique in demonstrating at a single cell level a relationship between the loss of CBP function and cell toxicity.

TUNEL assays detect DNA damage that can be a hallmark of both apoptotic and necrotic cell death (48). Numerous methods have been used to quantify cell death in transfected cell populations, some of which rely on the reduction in protein levels or activity [e.g. green fluorescence protein (29), firefly luciferase (49)] expressed from cotransfected plasmids. In order to provide an independent verification that polyQ-expanded htt is indeed inducing cell death, HT22 cells were cotransfected with a luciferase reporter plasmid and Htt-N63-19Q or Htt-N63-99Q expression plasmids. As shown in Figure 13, luciferase activity in HT22 cells expressing Htt-N63-99Q was only 10% of that recovered in cells expressing Htt-N63-19Q. Therefore, two independent assessments of cell death reveal Htt-N63-99Q-induced toxicity in HT22 cells, either at the level of individual cells (Figs 11 and 12) or transfected cell populations (Fig. 13).

**DISCUSSION**

**CBP is a target of polyQ-expanded proteins**

A number of studies have implicated the CBP coactivator as a selective target of polyQ-expanded proteins (29,33). CBP is essential for neuronal cell survival through its impact on various transcription factors with established pro-survival effects in neurons, such as the CRE-binding protein, CREB (31,32). Upon its recruitment into nuclear aggregates containing polyQ-expanded protein, CBP function is disrupted, leading to reductions in transcription from CREB target genes (29,39). Affymetrix array analysis has identified a number of candidate CREB-responsive genes whose expression is altered by expression of polyQ-expanded htt (39). The fact that CBP overexpression (29,30) or elevations in cAMP (39) could rescue polyQ-expanded htt or androgen receptor-mediated toxicity provides strong support for the notion that CBP can be limiting for neuronal cell survival. CBP, along with other coactivators, exerts its affects on transcription through the recruitment of histone acetyltransferases (50). The relevance of this CBP activity for neuronal cell survival is suggested by the observed inhibition of polyQ protein-dependent toxicity when histone acetylation is restored upon inhibition of histone deacetylases (51).

**HT22 cell toxicity is associated with nuclear localization of polyQ-expanded htt**

Nuclear localization of polyQ-expanded proteins appears to be important for triggering neuronal cell death (15,17,18,52,53), although in some cases polyQ-expanded proteins localized within the cytoplasm can also contribute to toxicity (8,54,55). In the HT22 hippocampal cell line, we find that toxicity within individual cells induced by polyQ-expanded htt, as revealed by a TUNEL assay, is associated with either nuclear or perinuclear aggregates. HT22 cells that contain cytoplasmic polyQ-expanded htt aggregates exhibit minimal signs of cell death under our culture conditions. Likewise, CBP loss or its recruitment into polyQ-expanded htt aggregates only occurs in HT22 cells containing nuclear or perinuclear aggregates. These results suggest that, as in other cell types examined (29),
Disruption of CBP function in an important determinant of polyQ-expanded htt toxicity.

PolyQ-expanded htt can enhance CBP processing by the UPP

While CBP recruitment into polyQ-expanded htt aggregates is one mechanism that serves to limit its function, we show here that loss of CBP protein, probably via degradation, could provide another mechanism that contributes to polyQ-expanded htt toxicity. In previous studies, CBP was found to co-localize with polyQ-expanded disease proteins in nuclear aggregates (27,29,33). Recruitment of CBP into polyQ-expanded protein aggregates has also been observed in brain tissue from polyQ repeat disease patients and transgenic mouse models of these diseases (27,29,33). While CBP is also recruited into polyQ-expanded htt protein aggregates in HT22 cells, CBP degradation appears to accompany the formation of nuclear htt aggregates in a relatively large fraction of HT22 cells (i.e. up to 40%). The loss of CBP staining was also observed in another neuroblastoma cell line (i.e. N2a cells) expressing htt nuclear aggregates and thus is not strictly a property of HT22 cells. Our results do not definitively exclude the possibility of polyQ-expanded htt effects on CBP gene expression, but two independent assessments of UPP action on CBP (see below) support a role for enhanced degradation contributing to CBP loss.

Aggregation of polyQ-expanded proteins within cells has been found to reduce proteasome function and contribute to toxicity (38,54,57). However, we find that in many cells expressing htt aggregates, CBP expression is reduced, owing in part to its degradation by the UPP. The recovery of CBP expression by proteasome inhibition in polyQ-expanded htt expressing cells supports this notion. Thus in addition to acting to block proteasome activity through the recruitment of essential proteasome components into nuclear aggregates (14,38,56), polyQ-expanded proteins may also specifically enhance proteasome-dependent degradation of select substrates (e.g. CBP). This possibility could reflect increased activity of some component of the UPP or altered conformation of polyQ-bound CBP leading to engagement of the ubiquitylating enzymes or the proteasome. In fact, we find increased CBP ubiquitylation in HT22 cells expressing polyQ-expanded htt. Thus, some fraction of CBP appears to be more effectively engaged with the UPP in cells expressing polyQ-expanded htt.

CBP degradation was observed in cells that express Htt-N63-99Q nuclear aggregates, although we cannot rule out the possibility that enhancement of proteasome activity can accompany the accumulation of toxic polyQ-expanded proteins that are not visibly associated with aggregates (17,18). Furthermore, the toxic form of polyQ-expanded htt may be an unfolded intermediate (58) that alters the conformation of associated CBP and thereby enhances its targeting to the UPP prior to the formation of visible aggregates.

Expression of polyQ-expanded htt can have a positive impact on protein degradation through an enhancement of proteasome subunit expression (59) or stimulation of endosomal-lysosomal activity (60). In differentiated N2a neuroblastoma cells, ubiquitin conjugation can be detected on both normal and polyQ-expanded htt, although proteasome-mediated degradation of the ubiquitylated polyQ-expanded htt is selectively impaired (56). Thus, it is conceivable that the UPP could be
activated in cells expressing polyQ-expanded htt but restricted in its action towards substrates (e.g. CBP) that are efficiently targeted to proteasomes following their ubiquitylation. While there is a reduction (i.e. ~40%) in soluble proteasome activity in differentiated N2a cells expressing htt with an expansion containing 150 glutamines, proteasome activity in a pelleted fraction is dramatically enhanced (i.e. ~10-fold) upon expression of this polyQ-expanded htt (56). Within an organelle (i.e. the nucleus) where proteasome action may typically be limited (56,61), any recruitment of proteasome subunits could enhance the degradation of potential substrates, prior to the eventual limitation of proteasome function through their sequestration into polyQ protein aggregates.

Not all nuclear proteins may share sensitivity to presumed activation of the UPP by expanded polyQ htt, as exhibited in our studies by the apparent lack of effects of polyQ-expanded htt nuclear aggregates on SP1 localization and expression. PolyQ-expanded htt effects on SP1 function do not require UPP involvement, but rather are due to a sequestration of SP1 from productive interactions with both its cognate DNA recognition sites and the TAFII130 transcription factor (62). Furthermore, the enhancement of CBP ubiquitylation is selective since polyQ-expanded htt did not affect ubiquitylation of another proteasome substrate in HT22 cells, i.e. the GR protein. Since pharmacological inhibition of the proteasome increased both GR and CBP ubiquitylation in HT22 cells, the lack of enhanced GR ubiquitylation in cells expressing polyQ-expanded htt demonstrates that proteasome function is not uniformly inhibited upon expression of polyQ-expanded htt. In a transgenic mouse model of SCA1, turnover of the protein kinase C delta isoform was increased in Purkinje cells of the cerebellum (63). In contrast, cerebellar Purkinje cell expression of the mGluR1 metabotropic glutamate receptor subunit protein was not affected in the SCA1 mice (63). Thus, selective enhancement of protein degradation may be a feature of other polyglutamine diseases and contribute at various levels to disease progression.

Cell culture and transgenic models of polyQ repeat diseases have been informative and provided important insights into the molecular mechanisms contributing to neuronal cell death in these diseases. However, given the protracted time course of polyQ repeat disease progression in humans, there are likely to be many biochemical changes that occur over many years that contribute to the selective neuronal cell vulnerability that is characteristic of these diseases. The UPP is clearly critical to the cell function at many levels and therefore requires precise regulation to maintain its appropriate action. As shown in this report, opposing effects of polyQ-expanded htt on proteasome action can ultimately have comparable detrimental effects on neuronal cell survival. The ubiquitin–proteasome pathway

Figure 10. Selective enhancement of endogenous CBP ubiquitylation in Htt-N63-99Q expressing HT22 cells. HT22 cells were transfected and an HA-tagged ubiquitin expression plasmid and either Htt-N63-19Q or Htt-N63-99Q expression plasmids. Where indicated, 5 μM lactacystin was added to transfected cultures for 24h. Soluble extracts from transfected cells were subjected sequentially to an immunoprecipitations with first, an anti-CBP antibody (A) and then an anti-GR (B) antibody. Immunoprecipitated proteins were subjected to western blot analysis to detected HA-tagged ubiquitylated proteins (A and B), or endogenous CBP (A) or GR (B) in the identical samples on parallel blots.
could therefore be involved at many levels during the progression of chronic neurodegenerative diseases.

MATERIALS AND METHODS

Plasmids

The htt (i.e. Htt-N63-19Q and Htt-N63-99Q) and CBP expression vectors used in these studies have been described previously (10,29). The HA-tagged ubiquitin plasmid (64) was obtained from Dirk Bohmann (University of Rochester).

Cell culture and transfection

Mouse HT22 cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA, USA) and penicillin/streptomycin (BioWhittacker, Walkersville, MD, USA). Mouse neuroblastoma 2a (N2a) cells (American-type culture collection, ATCC, Manassas, VA, USA) were maintained in minimum essential medium Eagle (ATCC) supplemented with 10% Vitacell FBS (ATCC) and penicillin/streptomycin. Cells were grown on coverslips in 6-well plates (Corning Incorporated Life Sciences, Acton, MA, USA) to ~50% confluence at the time of transfection. Transfections were performed using lipofectamine with cells maintained in serum free medium as recommended by the supplier (Invitrogen). Following 5h of exposure to the DNA-lipofectamine mixture, cells were refed with medium containing 10% FBS. Following an additional 24–48h incubation, cells were fixed and processed for IIF. Lactacystin (Sigma Chemicals, St Louis, MO, USA), when used, was added 18 h after transfection to attain a final concentration of 5 μM. In this case, cells were harvested after an additional 24 h incubation.

Antibodies and indirect immunofluorescence

Cells grown on glass coverslips were fixed and permeabilized using 2% paraformaldehyde (Sigma) and 0.1% Triton X-100 (ICN Biomedicals, Costa Mesa, CA, USA) in PBS, pH 7.4. Fixed cells were incubated with primary antibodies diluted in PBS plus 10% goat serum at appropriate concentrations for 1–1.5h at 37°C. The rabbit anti-c-myc A-14 antibody (Santa Cruz Biologicals, Santa Cruz, CA, USA) was used at a 1:200 dilution, the mouse anti-CBP A-22 antibody (Santa Cruz Biologicals) at 1:50, mouse anti-Sp1 1C6 antibody (Santa Cruz Biologicals) at 1:50 and mouse anti-ubiquitin antibody (Santa Cruz Biologicals) at 1:100. Following primary antibody incubation, cells were then washed and incubated with Rhodamine anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:250 and FITC antimouse IgG (Chemicon International, Temecula, CA, USA) at 1:400, and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) at 1:1000 for 1 h at 37°C before mounting. Coverslips were mounted onto slides using vectashield-mounting medium (Vector laboratories, Burlingame, CA, USA). IIF staining was examined using conventional fluorescence microscope. Approximately 100 transfected cells were counted in each experiment. Data are presented as
mean ± SEM of at least three separate experiments with statistical analysis performed using Student’s t-test.

Immunoprecipitation and western blot analysis

Protein extracts from transfected HT22 cells were prepared by lysis in buffer containing 50 mM Tris-Cl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1% NP-40 and a protease inhibitor cocktail (Sigma). Following a 5 min centrifugation of the extract at 14,000 g at 4°C, detergent-soluble supernatant and detergent-insoluble pellet fractions were collected. The insoluble fraction was solubilized with an equal volume of 2x SDS-sample buffer (1× = 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 20% β-mercaptoethanol, 2% SDS) prior to loading onto gels while the soluble fraction was adjusted to a final concentration of 1× in SDS-sample buffer prior to loading. Unless specifically indicated, identical amounts of total protein were loaded per well. Western blot analysis was performed essentially as described previously (65). Primary antibodies used were the mouse anti-CBP A-22 antibody (Santa Cruz Biologicals) at 1:100 dilution, the BuGR2 anti-GR monoclonal antibody (Affinity Bioreagents, Golden, CO, USA) at 1:2000 dilution, the mouse anti-c-Myc 9E10 antibody (Santa Cruz Biologicals) at 1:200 dilution or the goat anti-endogenous CBP or endogenous GR.

Figure 12. Quantitative analysis of cell death by TUNEL assay in HT22 cells expressing Htt-N63-99Q aggregates. Data are from the average of four independent experiments. Approximately 100 cells containing Htt-N63-99Q aggregates were counted from randomly selected fields in each experiment.

Figure 13. Cell death induced by Htt-N63-99Q. Viability of HT22 cells was assessed by measuring luciferase activity derived from a CMV-luciferase reporter plasmid co-transfected (at a 1:20 ratio) with either Htt-N63-19Q or Htt-N63-99Q plasmids. Data shown are average (±SEM) of four independent experiments (*P < 0.05).

Cell viability—luciferase assay

HT22 cells were transiently co-transfected with 0.05 μg of a pCMV-luciferase plasmid (66) and 1 μg of Htt-N63-19Q or 99Q plasmid in 35 mm tissue culture plates. Cells were harvested and lysed 48 h following transfection in Luciferase Cell Culture Lysis Buffer (Promega, Madison, WI, USA). Twenty microliters of supernatant collected after centrifugation of the lysate were used to measure luciferase activity using the Luciferase Assay Reagent (Promega) as described previously (66). Relative light units were normalized in each sample to total protein concentration.

ACKNOWLEDGEMENTS

D. Schubert (Salk Institute) is thanked for the kind gift of HT22 cells and Dirk Bohmann for the HA-tagged ubiquitin plasmid. The help of Dr Xinjia Wang with the immunoprecipitation assays is much appreciated. This work was supported by grants from the NIH (NS38319) and the Scaife Family Foundation to D.B.D. and by grants from the NIH (NS16375), Huntington’s Disease Society of America and the Hereditary Disease Foundation to C.A.R.

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