

Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation

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Loss of neurons in neurodegenerative diseases is usually preceded by the accumulation of protein deposits that contain components of the ubiquitin/proteasome system. Affected neurons in Alzheimer's disease often accumulate UBB⁺¹, a mutant ubiquitin carrying a 19–amino acid C-terminal extension generated by a transcriptional dinucleotide deletion. Here we show that UBB⁺¹ is a potent inhibitor of ubiquitin-dependent proteolysis in neuronal cells, and that this inhibitory activity correlates with induction

of cell cycle arrest. Surprisingly, UBB⁺¹ is recognized as a ubiquitin fusion degradation (UFD) proteasome substrate and ubiquitinated at Lys²⁹ and Lys⁴⁸. Full blockade of proteolysis requires both ubiquitination sites. Moreover, the inhibitory effect was enhanced by the introduction of multiple UFD signals. Our findings suggest that the inhibitory activity of UBB⁺¹ may be an important determinant of neurotoxicity and contribute to an environment that favors the accumulation of misfolded proteins.

Introduction

A broad array of human neurodegenerative diseases share strikingly similar histopathological features that may hold the key to their molecular pathogenesis (Sherman and Goldberg, 2001). A common finding is the presence of insoluble proteinaceous deposits, such as the neurofibrillary tangles and neuritic plaques of Alzheimer's disease, the Lewy bodies of Parkinson's disease, and the intranuclear inclusions of Huntington's disease, that differ in their protein content but invariably contain components of the ubiquitin/proteasome system (Schwartz and Ciechanover, 1999). As this cellular proteolytic machinery is involved in the clearance of misfolded proteins, this has led to the suggestion that a chronic imbalance between their generation and processing may be the primary cause for the formation of protein deposits (Cummins et al., 1998; Sherman and Goldberg, 2001). This model is further supported by the identification of inactivating mutations in a ubiquitin ligase (Kitada et al., 1998) and a deubiqui-

tinating enzyme (Leroy et al., 1998) as the cause for rare familial forms of Parkinson's disease as well as genetic mouse models of neurodegeneration (Saigoh et al., 1999). Moreover, the cellular toxicity correlated with nuclear inclusions can be suppressed by components of the ubiquitin/proteasome system (Fernandez-Funez et al., 2000), confirming the role of this proteolytic pathway in the clearance of their precursors.

The demonstration that components of the ubiquitin/proteasome system often are involved in neurodegeneration prompted us to examine whether a general impairment of the proteolytic machinery may contribute to the pathology. Recently, an aberrant form of ubiquitin was found in affected neurons of patients with different tauopathies such as sporadic and familial Alzheimer's disease, Down syndrome (van Leeuwen et al., 1998), progressive supranuclear palsy (Fergusson et al., 2000), Pick's disease, frontotemporal dementia, argyrophilic grain disease, and the polyglutamine disorder Huntington's disease (unpublished data), but not in synucleinopathies, such as Lewy body disease and multi-system atrophy (van Leeuwen et al., 1998). Ubiquitin is generated from precursor proteins consisting of tandem ubiquitin moieties that are cleaved into monomeric ubiquitin by ubiquitin C-terminal hydrolases (Wilkinson, 2000). Due to a mechanism known as molecular misreading (van Leeuwen

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et al., 2000), a dinucleotide deletion can occur within the mRNA encoding the ubiquitin B precursor resulting in a +1 frame shift close to the C terminus of the first ubiquitin moiety (van Leeuwen et al., 1998). Translation of the shifted open reading frame results in the product UBB^{+1} that comprises the first ubiquitin moiety with a 19-amino acid extension. Because the cleavage site of the ubiquitin C-terminal hydrolase is absent in UBB^{+1} , the extension is not removed. The aberrant C terminus prevents the activation and conjugation of UBB^{+1} , but due to the unaffected lysine residues, the mutant ubiquitin may serve as a scaffold for ligation of wild-type ubiquitin molecules (van Leeuwen et al., 2000). Synthetically ubiquitinated UBB^{+1} was shown to inhibit proteasomal degradation in vitro, and therefore it was hypothesized that its expression in neurons may disturb ubiquitin-dependent proteolysis (Lam et al., 2000). Using two different green fluorescent protein (GFP)*-based reporters that allow monitoring of ubiquitin-/proteasome-dependent proteolysis in living cells (Dantuma et al., 2000b), we show that UBB^{+1} acts as a strong inhibitor of the proteasome in vivo and induces a general accumulation of ubiquitinated substrates and cell cycle arrest. Surprisingly, UBB^{+1} is recognized as a ubiquitin fusion degradation (UFD) substrate and accordingly ubiquitinated at both Lys²⁹ and Lys⁴⁸ residues of its ubiquitin moiety. The inhibitory capacity relies on its recognition as a UFD substrate, as substitutions of either lysine residue releases the blockade while the inhibitory activity is further activated by enhancement of the UFD signal.

Results

UBB^{+1} inhibits the ubiquitin/proteasome system in living cells

Two previously characterized GFP-based proteasome substrates carrying an N-end rule (Ub-R-GFP) or a UFD (Ub^{G76V}-GFP) degradation signal (Dantuma et al., 2000b) were used to monitor ubiquitin-/proteasome-dependent proteolysis in UBB^{+1} -expressing cells. The N-end rule degradation signal triggers ubiquitination close to the N terminus of the GFP reporter once the ubiquitin moiety of the fusion is cleaved by endogenous ubiquitin C-terminal hydrolases (Varshavsky, 1996), whereas the UFD signal includes the N-terminal uncleavable ubiquitin moiety Ub^{G76V} that serves as target for polyubiquitination (Johnson et al., 1995). Because UBB^{+1} mainly has been found in neurons, the reporters were stably transfected in the SH-SY5Y neuroblastoma cell line. In addition, we used a previously characterized HeLa transfectant that constitutively expresses the Ub^{G76V}-GFP reporter (Dantuma et al., 2000b). Reporter-expressing SH-SY5Y and HeLa cells were transiently transfected with FLAG-tagged ubiquitin (^{FLAG}Ub) or UBB^{+1} , and were analyzed in parallel for expression of these proteins and activity of the ubiquitin/proteasome system as assessed by accumulation of the GFP fluorescence. Microscopic and flow cytometric analysis revealed accumulation of the Ub^{G76V}-GFP and Ub-R-GFP reporters in cells expressing de-

tectable amounts of UBB^{+1} , whereas overexpression of ^{FLAG}Ub had no effect (Fig. 1 A). Flow cytometric analysis of HeLa cells that accumulated the Ub^{G76V}-GFP reporter revealed a 60-fold increased fluorescence intensity (unpublished data), compared with a 100-fold increase in the same assay after treatment with potent inhibitors of the proteasome (Dantuma et al., 2000b; Myung et al., 2001). It is noteworthy that even though the vast majority of UBB^{+1} -positive cells accumulated the Ub^{G76V}-GFP reporter, the percentage of fluorescent cells was ~1–2% of the total population, which is surprisingly low, as transfection efficiencies between 20 and 40% were routinely obtained in these HeLa cells (see below).

Kinetics of Ub^{G76V}-GFP accumulation in UBB^{+1} -positive cells showed that after 10 h, ~1/2 of the UBB^{+1} -expressing cells had elevated levels of the Ub^{G76V}-GFP proteasome substrate, which further increased to 80% at 20 h posttransfection (Fig. 1 B). Thus, the expression of UBB^{+1} preceded the accumulation of GFP. Only background fluorescence was detected in cells expressing ^{FLAG}Ub (Fig. 1 B). In order to study whether the elevated Ub^{G76V}-GFP steady state levels are due to delayed turnover of this proteasome substrate in response to UBB^{+1} , we evaluated the clearance of the accumulated proteasome substrate after blocking protein synthesis with cycloheximide. We would like to emphasize that the cycloheximide treatment will block not only the expression of Ub^{G76V}-GFP, but also of UBB^{+1} . To validate the experimental set up, we first tested the clearance of Ub^{G76V}-GFP from cells in which the GFP substrate had been accumulated during a short incubation with the reversible proteasome inhibitor MG132. Incubation with the proteasome inhibitor resulted in an ~10-fold induction of GFP fluorescence. After removing MG132 and blocking protein synthesis with cycloheximide the cells degraded the accumulated Ub^{G76V}-GFP within 4 h (Fig. 1 C). In sharp contrast, the mean fluorescence intensity of cells transfected with UBB^{+1} did not decline over a 6-h period, but rather showed a modest increase. Although the percentage GFP fluorescent cells declined in both the control and the UBB^{+1} -transfected cells, after 6 h, we observed that only in the UBB^{+1} -transfected cells was there still a substantial amount of cells with accumulated Ub^{G76V}-GFP (Fig. 1 D; unpublished data). These data show that the accumulated Ub^{G76V}-GFP has a prolonged half-life in cells transfected with UBB^{+1} . The decrease of GFP fluorescent cells upon blockage of protein synthesis in UBB^{+1} -transfected cells also suggests that newly synthesized proteins are required to maintain a full blockage on the ubiquitin/proteasome system.

Western blot analysis of UBB^{+1} -transfected HeLa and SH-SY5Y cells revealed the presence of unmodified UBB^{+1} , as well as three slower migrating bands (Fig. 1 E; unpublished data). This pattern corresponds to that found in earlier studies in which the bands were identified as conjugates of UBB^{+1} with one, two, or three ubiquitin moieties (Lam et al., 2000; de Vrij et al., 2001).

Expression of UBB^{+1} induces accumulation of polyubiquitinated proteins and cell cycle arrest

In subsequent experiments we analyzed the ubiquitination status of accumulating proteasome substrates in UBB^{+1} -expressing cells. Ub^{G76V}-GFP HeLa cells were transiently

*Abbreviations used in this paper: GFP, green fluorescent protein(s); nfGFP, nonfluorescent GFP; UFD, ubiquitin fusion degradation; Z-L₃-VS, carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone.

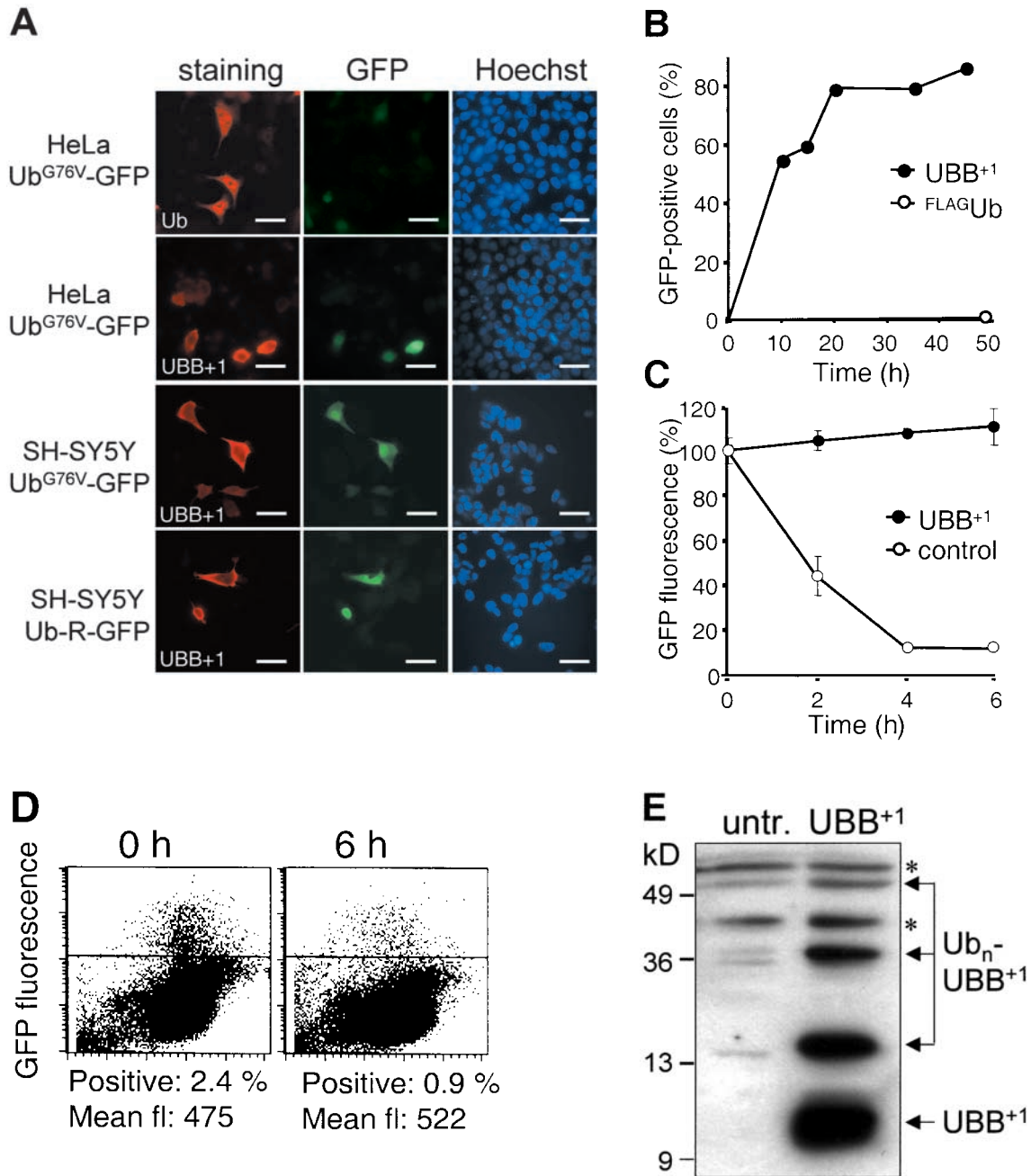
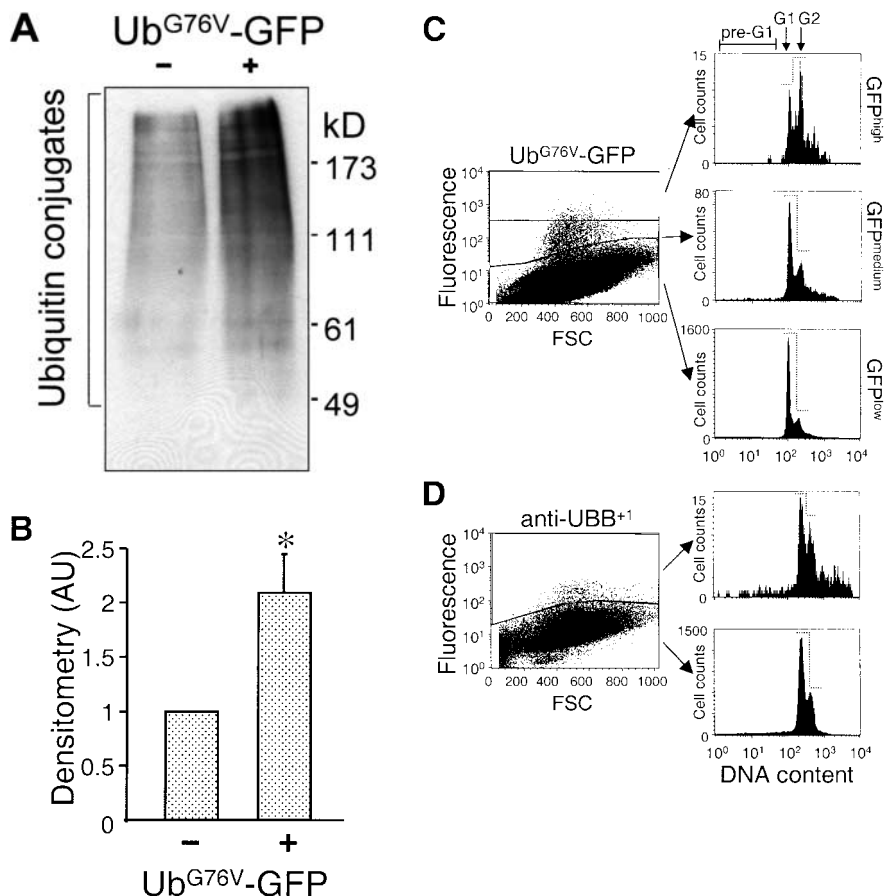


Figure 1. UBB⁺¹ inhibits the ubiquitin/proteasome pathway in vivo. (A) HeLa and SH-SY5Y cell lines stably expressing Ub^{G76V}-GFP or Ub-R-GFP were transfected with FLAGUb or UBB⁺¹. Cells were stained with an anti-FLAG or anti-UBB⁺¹ antibody and nuclei were counterstained with Hoechst 33258. Representative micrographs show expression of FLAGUb and UBB⁺¹ (left, red), fluorescence of Ub^{G76V}-GFP or Ub-R-GFP (middle, green), and counterstaining with Hoechst 33258 (right, blue). Bar, 20 μ m. (B) Ub^{G76V}-GFP HeLa cells were transfected with UBB⁺¹ or FLAGUb harvested at indicated time points and analyzed by fluorescence microscopy. The results are expressed as percentage of UBB⁺¹- or FLAGUb-positive cells accumulating Ub^{G76V}-GFP. (C) Protein synthesis was blocked in Ub^{G76V}-GFP HeLa cells transfected with UBB⁺¹ by administration of 50 μ g/ml cycloheximide. As a control, cycloheximide was added to Ub^{G76V}-GFP HeLa cells in which the reporter had been accumulated by a 2.5-h treatment with the reversible proteasome inhibitor MG132. The mean fluorescence intensity of the GFP fluorescent population was determined at the indicated time points by flow cytometry. Mean fluorescence when cycloheximide was administered was standardized as 100%. Triplicate values of representative experiment. (D) Flow cytometric analysis of Ub^{G76V}-GFP fluorescence in UBB⁺¹-transfected cells upon administration of cycloheximide at time points 0 and 6 h (as in C). Percentage GFP-positive cells and their mean fluorescence intensity are indicated. (E) Western blot analysis with an anti-UBB⁺¹ antibody of cell lysates of untransfected and UBB⁺¹ transfected HeLa cells. Molecular mass marker and bands corresponding to UBB⁺¹ and ubiquitinated UBB⁺¹ are indicated. (*) Nonspecific immunoreactive bands.

transfected with UBB⁺¹ and then sorted by flow cytometry based on GFP fluorescence intensity. Western blots of lysates from GFP-positive and -negative cells probed with an

anti-ubiquitin antibody demonstrated that elevated GFP levels correlated with a general accumulation of polyubiquitinated proteins (Fig. 2 A), corresponding to an approxi-

Figure 2. UBB⁺¹ induces accumulation of polyubiquitinated proteins and G2/M cell cycle arrest. (A) Ub^{G76V}-GFP HeLa cells were transfected with UBB⁺¹ and 40,000 high fluorescent and 40,000 low fluorescent cells were sorted by flow cytometry 48 h posttransfection. Cell lysates of these populations were analyzed by Western blot probed with an anti-ubiquitin antibody. Molecular mass marker is indicated. (B) Quantitative analysis of anti-ubiquitin immunoreactivity by densitometry from three independent experiments as described in A. (C) Flow cytometry analysis of Ub^{G76V}-GFP HeLa cells transiently transfected with UBB⁺¹ on the left. The cell cycle distribution, analyzed by propidium iodide staining, of the Ub^{G76V}-GFP^{high}, Ub^{G76V}-GFP^{medium}, and Ub^{G76V}-GFP^{low} fluorescence are illustrated to the right. One representative experiment out of three. (D) Flow cytometric analysis of UBB⁺¹-transfected parental HeLa cells stained with an anti-UBB⁺¹ antibody (left). The cell cycle distribution of the UBB⁺¹-positive and negative population is shown (right).



mately twofold increase in the intensity of the smear of polyubiquitin adducts (Fig. 2 B). Thus, UBB⁺¹ is likely to affect an event downstream of polyubiquitination.

Impairment of the ubiquitin/proteasome system accompanied by the accumulation of polyubiquitinated proteins, as observed in cells treated with inhibitors of the proteasome, normally results in induction of apoptosis often preceded by arrest in the G2/M phase of the cell cycle (Lee and Goldberg, 1998; Dantuma et al., 2000b). Therefore, 48 h posttransfection we analyzed the cell cycle distribution of UBB⁺¹-transfected Ub^{G76V}-GFP HeLa cells emitting background (Ub^{G76V}-GFP^{low}), moderately elevated (Ub^{G76V}-GFP^{medium}) or high levels of GFP fluorescence (Ub^{G76V}-GFP^{high}). Ub^{G76V}-GFP^{low} cells displayed a cell cycle distribution comparable to that of untransfected cells, whereas a larger proportion of the Ub^{G76V}-GFP^{medium} and Ub^{G76V}-GFP^{high} cells were found in the G2/M phase, which is indicative for cell cycle arrest (Fig. 2 C). A similar G2/M arrest was observed in parental HeLa cells expressing UBB⁺¹, excluding the possibility that accumulation of the GFP reporter may be responsible for the effect (Fig. 2 D). Within the time frame of our transient transfection, we did not observe a significant increase of apoptotic cells in the GFP positive populations.

UBB⁺¹ is a UFD substrate

To test whether physiological ubiquitination is required for the inhibitory activity of UBB⁺¹ *in vivo*, we generated the mutant UBB^{+1/K48R} in which the common ubiquitin conjugation site

Lys⁴⁸ was substituted with Arg. Surprisingly, UBB^{+1/K48R} was still subject to ubiquitination in SH-SY5Y and HeLa cells (Fig. 3 A; unpublished data), suggesting that an alternate ubiquitination site may be used. Targeting of substrates for proteasomal degradation may also occur via the less common ubiquitination site Lys²⁹. To date, this site has only been described for UFD substrates in yeast in which both Lys²⁹ and Lys⁴⁸ of the N-terminal ubiquitin moiety are targets for polyubiquitination (Johnson et al., 1995; Koegl et al., 1999). Therefore, we compared UBB⁺¹ mutants carrying Lys²⁹→Arg and Lys⁴⁸→Arg substitutions. Indeed, both UBB^{+1/K29R} and UBB^{+1/K48R} were equally efficiently ubiquitinated, whereas ubiquitin conjugation was virtually abrogated in the double mutant UBB^{+1/K29,48R} (Fig. 3 A). Furthermore, substitution of either lysine residue was sufficient to induce a significant increase in the steady state levels of the mutant protein. The effect was most dramatic with the UBB^{+1/K29R} mutant (Fig. 3 A), suggesting that this ubiquitination site may preferentially target UBB⁺¹ for proteasomal degradation. Surprisingly, we observed consistently higher levels of UBB^{+1/K29R} as compared with UBB^{+1/K29,48R} in both HeLa and neuroblastoma cells. Although we did not fully understand this observation, subsequent analysis confirmed that this is not due to proteasomal degradation of the double mutant (Fig. 3 C; unpublished data).

Paradoxically, we observed that the UBB⁺¹ is a potent inhibitor of the ubiquitin/proteasome system, whereas proteins carrying a UFD signal are normally rapidly degraded by the proteasome (Johnson et al., 1992, 1995). As noted above, we observed in transient transfections an unanticipated low per-

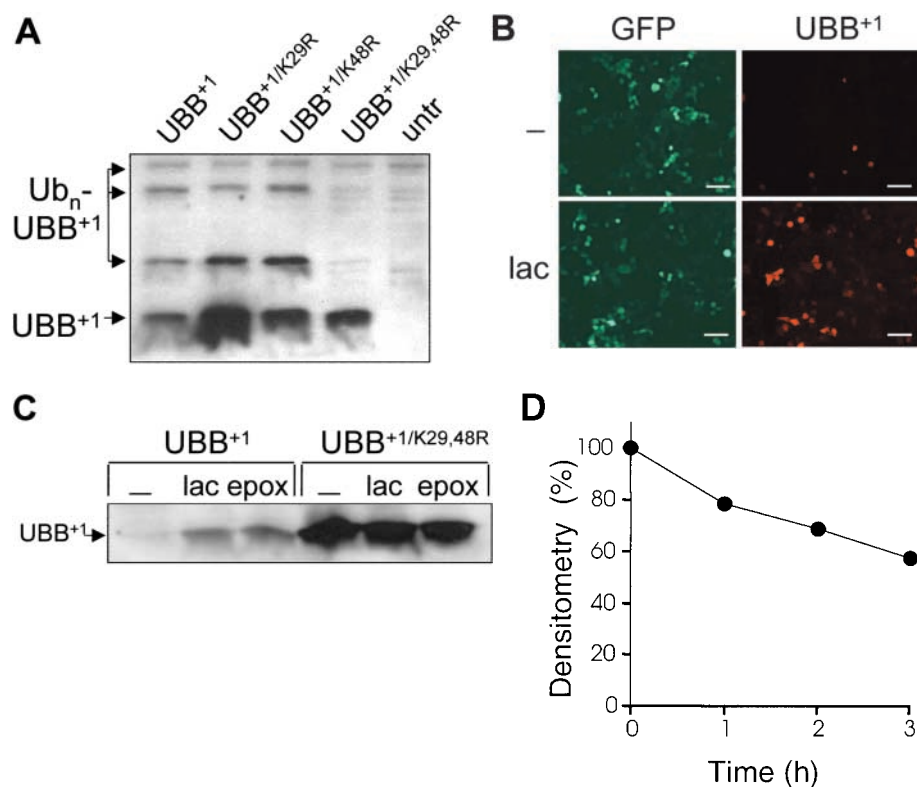


Figure 3. UBB⁺¹ is a UFD substrate. (A) Western blot analysis with an anti-UBB⁺¹ antibody of cell lysates from HeLa cells transfected with UBB⁺¹, UBB^{+1/K29R}, UBB^{+1/K48R}, UBB^{+1/K29,48R}. Products corresponding to unmodified and ubiquitinated UBB⁺¹ are indicated. (B) Micrographs of HeLa cells transfected with pCMS-UBB⁺¹/GFP that were left untreated (top) or incubated for 16 h with 30 μ M lactacystin (bottom). Transfected cells were identified by GFP expression (left) and transfected cells expressing detectable levels of UBB⁺¹ were visualized by immunostaining (right). Bars, 100 μ m. (C) Western blot analysis with an anti-UBB⁺¹ antibody of the steady-state levels of UBB⁺¹ and UBB^{+1/K29,48R} in transiently transfected HeLa cells that were left untreated or incubated the proteasome inhibitors lactacystin (30 μ M) or epoxomicin (500 nM). (D) The turnover of UBB⁺¹ was determined by pulse-chase analysis in SK-N-SH neuroblastoma cells transduced with lenti-UBB⁺¹. Intensity of the UBB⁺¹ band was quantified with a phosphoimager and the intensity at time point 0 was standardized as 100%. (A–D) One representative experiment out of three.

centage of cells with detectable levels of the UBB⁺¹ protein. This prompted us to investigate the possibility that the UBB⁺¹ may be degraded in a fraction of the cells. To this end, we constructed a plasmid in which UBB⁺¹ expression and GFP expression are driven by the CMV and SV40 promoters, respectively, which allowed us to identify all transfected cells by the GFP fluorescence. Microscopic examination showed that only \sim 5% of the transfected cells expressed detectable amounts of UBB⁺¹ (Fig. 3 B, top). Inclusion of the specific proteasome inhibitor lactacystin (Fig. 3 B, bottom) or epoxomicin (unpublished data) resulted in accumulation of UBB⁺¹ in a great part of transfected cells. Western blot analysis confirmed the increase of UBB⁺¹ in response to lactacystin and epoxomicin and showed that proteasomal degradation of UBB⁺¹ was abrogated when Lys²⁹ and Lys⁴⁸ were substituted with Arg residues (Fig. 3 C). Pulse-chase analysis of neuroblastoma cells transduced with a lentiviral vector encoding UBB⁺¹ revealed that the UBB⁺¹ levels declined over the 3-h period monitored, which is in line with the notion that UBB⁺¹ is degraded in many cells (Fig. 3 D). These data, together with the experiment shown in Fig. 1, C and D, indicate that whereas the fast majority of UBB⁺¹-expressing cells turnover the mutant ubiquitin, it remains stable in a fraction of the cells due to a general blockage of the ubiquitin/proteasome system. Therefore, we conclude that UBB⁺¹ is an authentic UFD substrate and degraded accordingly by the ubiquitin/proteasome system in many cells.

Ubiquitination as a UFD substrate is required for a full inhibitory activity

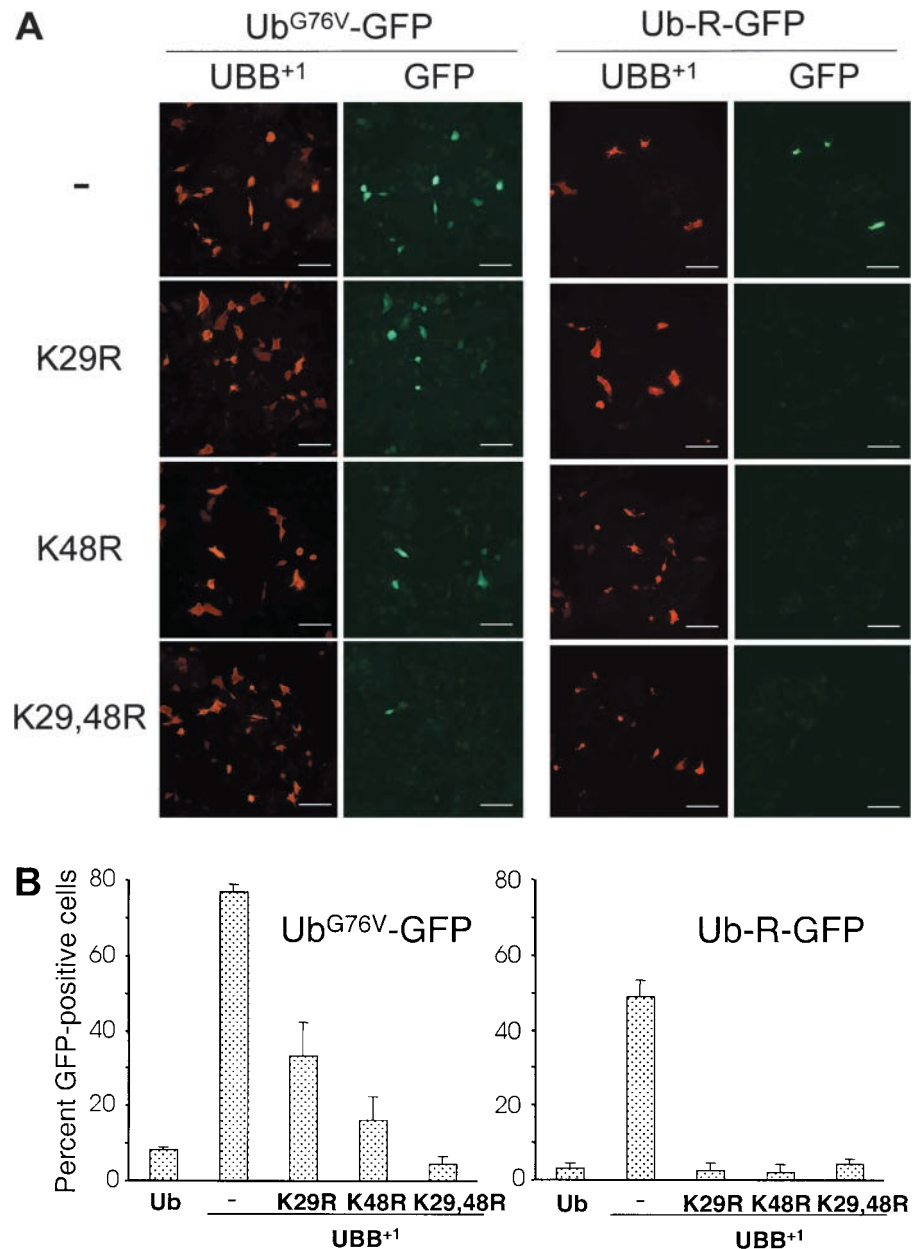
Next, we tested whether ubiquitination at specific sites is required for the inhibitory activity of UBB⁺¹. UBB⁺¹ mutants

lacking the Lys²⁹, Lys⁴⁸, or both ubiquitination sites were transiently transfected in SH-SY5Y cells expressing the GFP reporters and the activity of the ubiquitin/proteasome system was monitored by measuring GFP accumulation. Mutation of both Lys²⁹ and Lys⁴⁸ abrogated the accumulation of both GFP reporters in the neuroblastoma cells confirming that ubiquitination is critical for the inhibitory effect (Fig. 4, A and B). Surprisingly, substitutions of single lysine residues had different effects on the degradation of UFD and N-end rule substrates. The single lysine mutants UBB^{+1/K29R} and UBB^{+1/K48R} were still able to inhibit the degradation of Ub^{G76V}-GFP, although the inhibitory effect was strongly compromised. In contrast, substitution of either lysine residue was sufficient to fully abrogate the effect of UBB⁺¹ on accumulation of the Ub-R-GFP reporter, demonstrating that both ubiquitination sites are required to block the degradation of N-end rule substrates. Thus, efficient inhibition of the ubiquitin/proteasome system can only be accomplished by UBB⁺¹ containing both ubiquitination sites.

Lys²⁹ or Lys⁴⁸ residues can independently target an authentic UFD substrate for degradation

The intriguing finding that UBB⁺¹ needs both lysine residues for optimal inhibitory activity brought up the question whether these two ubiquitination sites act in concert or independently in targeting substrates to the proteasome. This question is difficult to address with UBB⁺¹, as the different UBB⁺¹ mutants with lysine substitutions were shown to differ in their capacity to inhibit the proteasome; therefore, changes in the turnover of these mutants can be due to targeting as well as inhibitory events. For this reason we turned to the Ub^{G76V}-GFP reporter, which is a designed UFD substrate

Figure 4. Inhibitory activity of UBB⁺¹ requires ubiquitination at Lys²⁹ and Lys⁴⁸. (A) Micrographs of Ub^{G76V}-GFP (left) and Ub-R-GFP SH-SY5Y cells (right) transfected with UBB⁺¹, UBB^{+1/K29R}, UBB^{+1/K48R}, or UBB^{+1/K29,48R}. The cells were stained for UBB⁺¹ (left) and analyzed for GFP fluorescence (right). Bars, 100 μ m. (B) Quantification of three independent experiments as shown in A. The results are expressed as the percent of the UBB⁺¹ expressing cells with accumulated Ub^{G76V}-GFP or Ub-R-GFP levels.



that allows easy evaluation of proteasomal degradation (Dantuma et al., 2000b). We used a previously described flow cytometric assay in which HeLa cells were transiently transfected with the different Ub^{G76V}-GFP mutants and the percentage of GFP fluorescent cells in the absence or presence of the proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L₃-VS; Bogoy et al., 1997) was determined (Dantuma et al., 2000a). Substitution of both Lys²⁹ and Lys⁴⁸ residues in Ub^{G76V}-GFP completely abrogated proteasomal degradation of the GFP reporter (Fig. 5, A and B), confirming that these two lysines are the sole ubiquitination sites targeting for degradation. We observed that substitution of Lys²⁹ resulted in a partial stabilization, whereas removal of Lys⁴⁸ did not stabilize the protein. These data show that each of these two ubiquitin trees can function as an autonomous signal that target a model UFD substrate to the proteasome. Yet, similar to the situation in yeast (Johnson et al., 1995;

Koegl et al., 1999), the Lys²⁹ tree appears to be more effective than Lys⁴⁸ in targeting a UFD for degradation.

Enhancement of the UFD signal strengthens the inhibitory activity of UBB⁺¹

Because UBB⁺¹ is a target as well as an inhibitor of the ubiquitin/proteasome system, we asked whether the inhibitory activity could be reversed by modifications that may enhance its degradation. UFD signals can be turned into a more potent degradation signal by introducing multiple tandem organized uncleavable ubiquitin moieties (Stack et al., 2000). Therefore, we inserted one or two additional uncleavable ubiquitin (Ub*) moieties at the N terminus of UBB⁺¹ and generated the Ub*-UBB⁺¹ and Ub*₂-UBB⁺¹ constructs (Fig. 6 A). However, unexpectedly, enhancement of the UFD signal did not result in accelerated turnover of UBB⁺¹, as reported with other UFD substrates (Stack et al., 2000), but instead a dramatic accumu-

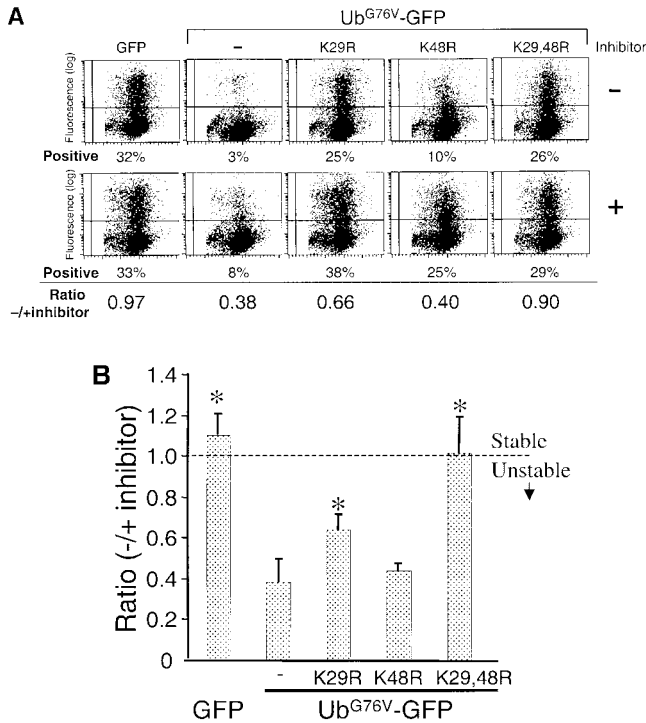


Figure 5. Lys²⁹ and Lys⁴⁸ can independently target a UFD substrate for degradation. (A) Dot plots of flow cytometric analysis of HeLa cells transiently transfected with GFP, Ub^{G76V}-GFP, Ub^{K29R/G76V}-GFP, Ub^{K48R/G76V}-GFP, and Ub^{K29,48R/G76V}-GFP. Half of the cells were left untreated and the other half was incubated for 16 h with 10 μM of the proteasome inhibitor Z-L₃-VS. The percentage GFP-positive cells and the ratio between the percentage of fluorescent cells in samples untreated/inhibitor-treated are indicated. (B) Quantification of three independent experiments as shown in A. Values significantly different from the Ub^{G76V}-GFP sample are marked with asterisks (*t* test, *P* < 0.05). Mean ± SD of three independent experiments. Ratios < 1 indicate proteasomal degradation of the protein.

lation of UBB⁺¹ was observed (Fig. 6 B). The effect was most apparent with Ub^{*₂}-UBB⁺¹, in which in addition high-molecular mass species were observed in the stacking gel, implying that polyubiquitin trees are conjugated to UBB⁺¹.

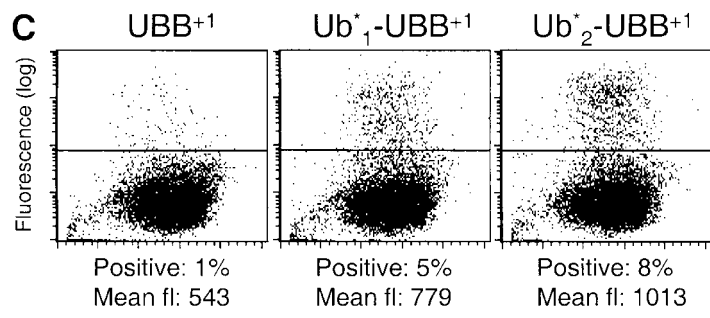
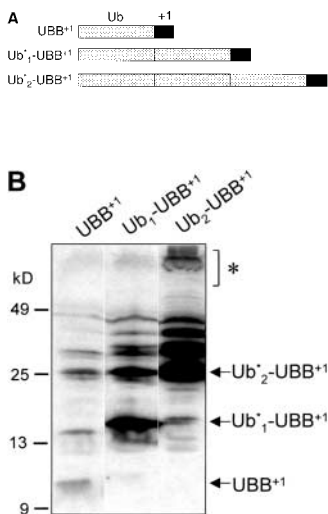


Figure 6. Targeting UBB⁺¹ for proteasomal degradation enhances its inhibitory effect. (A) Schematic illustration of the UBB⁺¹, Ub^{*₁}-UBB⁺¹, and Ub^{*₂}-UBB⁺¹ constructs. (B) Western blot analysis with anti-UBB⁺¹ antibody of cell lysates of HeLa cells transfected with UBB⁺¹, Ub^{*₁}-UBB⁺¹, Ub^{*₂}-UBB⁺¹. Molecular mass marker and bands corresponded to unmodified and ubiquitinated UBB⁺¹ proteins as well as high molecular mass UBB⁺¹ are indicated. (C) Flow cytometric analysis of GFP fluorescence of Ub^{G76V}-GFP HeLa cells transfected with UBB⁺¹, Ub^{*₁}-UBB⁺¹, and Ub^{*₂}-UBB⁺¹. The percentage of cells with accumulated GFP and the mean fluorescence intensity of this population are indicated at the bottom.

Next, we compared the effect of UBB⁺¹, Ub^{*₁}-UBB⁺¹, and the Ub^{*₂}-UBB⁺¹ on proteasomal degradation in HeLa and SH-SY5Y cells. In line with the positive correlation between the number of N-terminal ubiquitin moieties and the amounts of UBB⁺¹, Ub^{*₁}-UBB⁺¹, or Ub^{*₂}-UBB⁺¹ accumulating in transfected cells, we found a dose-dependent correlation between the number of ubiquitin moieties and the accumulation of Ub^{G76V}-GFP in HeLa cells (Fig. 6 C) and Ub^{G76V}-GFP and Ub-R-GFP in SH-SY5Y cells (unpublished data). Thus, targeting for ubiquitin-/proteasome-dependent degradation is crucial for the inhibitory activity of UBB⁺¹, and enhancement of its degradation signal paradoxically increases its stability and strengthens its inhibitory activity resulting in a more severe inhibition of proteasomal degradation.

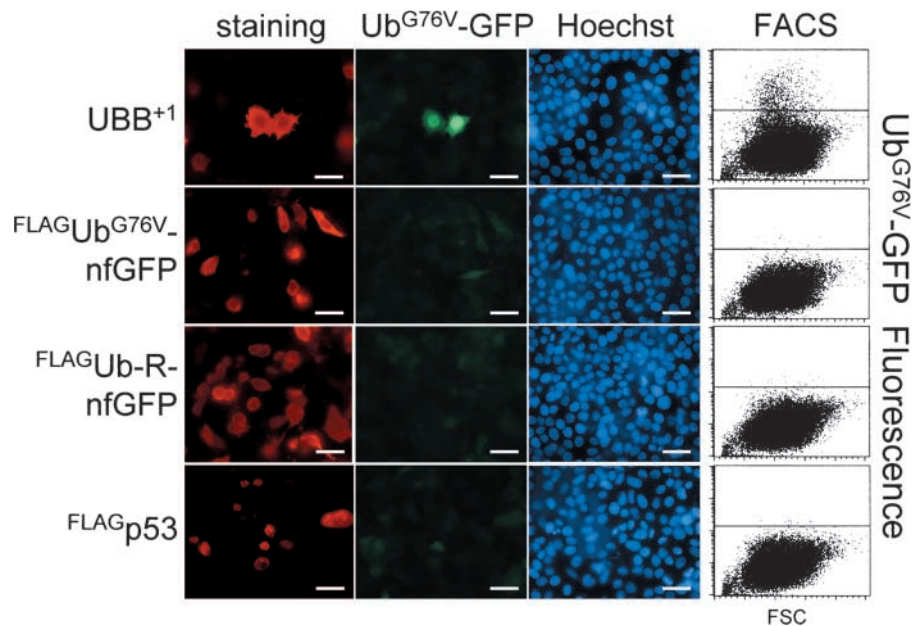
No impaired proteasomal degradation in response to overexpression of other substrates

A possible explanation for the inhibitory activity of UBB⁺¹ is that overexpression of proteasome substrates will saturate the system and competitively affect degradation of the Ub-R-GFP and Ub^{G76V}-GFP substrates. To address this issue, we designed substrates whose expression was driven by the CMV promoter similar to the UBB⁺¹ constructs. These substrates were FLAG-Ub-R-nfGFP and FLAG-Ub^{G76V}-nfGFP, which are based on a nonfluorescent variant of GFP (nfGFP), and FLAG-p53. Ub^{G76V}-GFP HeLa cells expressing the substrate were identified by the FLAG tag present on each of the substrates. Microscopic and flow cytometric analysis demonstrated that only UBB⁺¹ was able to block degradation of the GFP substrate, whereas none of the other three substrates had an effect on Ub^{G76V}-GFP levels (Fig. 7). It is noteworthy that even the nonfluorescent variant of the Ub^{G76V}-GFP substrate itself did not induce accumulation. Hence, the inhibitory effect of UBB⁺¹ is not simply due to saturating the ubiquitin/proteasome system by overexpression of a substrate.

Discussion

In the present study we show that an abnormal component of the ubiquitin/proteasome system, which has been detected in a broad variety of neurodegenerative diseases, can

Figure 7. Overexpression of other proteasome substrates does not inhibit turnover of Ub^{G76V}-GFP. Ub^{G76V}-GFP HeLa cells were transiently transfected with UBB⁺¹, FLAG-Ub^{G76V}-nfGFP, FLAG-Ub-R-nfGFP and FLAG-p53. UBB⁺¹ transfected cells were stained with the anti-UBB⁺¹ antibody while the nonfluorescent FLAG-Ub^{G76V}-nfGFP and FLAG-Ub-R-nfGFP constructs and FLAG-p53 were stained with a FLAG-specific antibody. Representative micrographs of the immunostaining (left, red), the Ub^{G76V}-GFP fluorescence (middle, green), and the Hoechst 33258 counterstaining (right, blue) are shown. Note that as expected the FLAG-Ub^{G76V}-nfGFP and FLAG-Ub-R-nfGFP give a homogenous staining in the cytosol and nucleus, whereas FLAG-p53 is localized in the nucleus. To the left are shown flow cytometric analysis of the GFP fluorescence upon transfection with the different constructs.



inhibit proteasomal degradation in neuronal cells. Interestingly, all the pathologic conditions for which expression of UBB⁺¹ has been described, including several tauopathies and a polyglutamine disorder, are characterized by the accumulation of insoluble deposits formed by aggregated proteins (Sherman and Goldberg, 2001). Under normal conditions, misfolded proteins are efficient substrates of ubiquitin-/proteasome-dependent proteolysis, and a key question has been the nature of the primary events that favors their accumulation rather than rapid clearance in affected neurons. Our data show that UBB⁺¹ is a powerful inhibitor of this proteolytic pathway *in vivo*. The effect was sufficient to induce cell cycle arrest at the G2/M boundary, at least under the conditions of overexpression achieved in our transient transfection assays. A particularly important aspect of our findings is the demonstration that UBB⁺¹ is not only an inhibitor, but also a target of the ubiquitin/proteasome system. Interestingly, it has been shown that whereas UBB⁺¹ transcripts are present in both normal and affected brains, the protein product has only been detected in affected neurons of individuals suffering from neurodegenerative disorders (unpublished data). Notably, we observed that only a small population of the transfected cells expressed detectable levels of the UBB⁺¹ protein followed by accumulation of the GFP substrates, whereas the majority of the cells destroy the UBB⁺¹ by proteasomal degradation. Using an adenovirus based transduction method in neurons, which accomplishes massive expression of UBB⁺¹, and an *in vitro* degradation assay, it was recently shown that UBB⁺¹ is a rather stable and toxic protein (de Vrij et al., 2001). Conceivably, the ubiquitin/proteasome system can cope with low levels of UBB⁺¹ but accelerated proteasomal targeting, by elevated steady-state levels or by enhancement of the UFD signal, obstructs ubiquitin-/proteasome-dependent proteolysis of this aberrant ubiquitin. Alternatively, the cells that accumulate UBB⁺¹ and the GFP substrates have a sub-optimal ubiquitin/proteasome system, making them more sensitive to the inhibitory effect of UBB⁺¹. We envision that

in vivo slight changes in the efficiency of proteolysis, as may be achieved in selected neurons by the production of β -amyloid peptide in Alzheimer's disease (Gregori et al., 1995; Keller et al., 2000), or the formation of insoluble aggregates in polyglutamine disorders (Bence et al., 2001; Jana et al., 2001), may be sufficient to initiate a process resulting in accumulation of UBB⁺¹ that will eventually lead to cellular intoxication by a general inhibition of the ubiquitin/proteasome system and ultimately to cell death.

Detailed analysis of the requirements for the inhibitory effect of UBB⁺¹ revealed some unexpected characteristics. It was acknowledged earlier that UBB⁺¹, even though it cannot be conjugated to substrates (van Leeuwen et al., 1998), can serve as a recipient for polyubiquitination. Therefore, it was postulated that polyubiquitinated UBB⁺¹, similar to free polyubiquitin trees (Piotrowski et al., 1997), can block proteolysis of proteasome substrates (Lam et al., 2000). Indeed, we confirm that ubiquitination of UBB⁺¹ is required for its inhibitory activity *in vivo*. However, several lines of evidence argue that ubiquitinated UBB⁺¹ does not simply act as a free polyubiquitin tree but is instead an aberrant UFD substrate. First, we show that UBB⁺¹ is ubiquitinated both at Lys²⁹ and Lys⁴⁸, a pattern that is unique for UFD substrates (Johnson et al., 1995; Koegl et al., 1999). Second, UBB⁺¹ is structurally similar to a UFD substrate, as it has an N-terminal uncleavable ubiquitin moiety linked to a C-terminal extension. Third, our data clearly demonstrate that UBB⁺¹ is degraded by the proteasome in a large number of the transfected cells.

Even though the related UFD reporter Ub^{G76V}-GFP seems to be susceptible to inhibition by UBB⁺¹ with a single ubiquitination site to some extent, blockage of degradation of the Ub-R-GFP reporter required the Lys²⁹ as well as Lys⁴⁸ residues. One possible explanation is that the pool of inhibitory UBB⁺¹ consists of molecules bearing two ubiquitin trees. Binding of both trees to acceptor sites in the proteasome may be required to achieve interactions sufficiently tight to prevent access to other polyubiquitinated substrates. It is noteworthy that in the crystal structure of ubiquitin the Lys²⁹ and

Lys⁴⁸ residues are localized on opposite faces of the molecule and would structurally allow double ubiquitin trees (Cook et al., 1994). It is also possible that the two sites act cooperatively in optimizing ubiquitination, as suggested by the recent finding that in yeast the polyubiquitination factor E4/UFD2 requires Lys⁴⁸ in a UFD signal in order to accommodate efficient polyubiquitination at Lys²⁹ (Koegl et al., 1999). Interestingly, a recombination event in the gene encoding the murine homologue of E4/UFD2 may underlie the delayed Wallerian nerve degeneration observed in a mouse strain (Conforti et al., 2000). The experiments with the Ub^{G76V}-GFP substrate strongly support the model based on a tight interaction between UBB⁺¹ bearing two ubiquitin trees and the proteasome, as both lysine residues can independently target this model UFD substrate to the proteasome, suggesting that Lys²⁹ and Lys⁴⁸ can each bear a functional ubiquitin tree. Several studies suggest that the rate of polyubiquitination determines the duration of the interaction between a substrate and the proteasome (Lam et al., 1997; Thrower et al., 2000), and it is likely that regardless of whether these two lysine residues are required for the formation of double ubiquitin trees or more efficient polyubiquitination at Lys²⁹, the outcome is a polyubiquitinated UBB⁺¹ that cannot be rapidly released from the proteasome. The combination of a tightly bound but poorly degradable proteasome substrate may clog the system by obstructing access to other substrates, especially when the UBB⁺¹ feed in large amounts to the proteasome. It is tempting to speculate that in its short C-terminal extension may lie the reason for the inhibitory activity of UBB⁺¹, either because it is too short to allow efficient tethering of the recruited UBB⁺¹ into the cavity of the proteasome as has been proposed for another UFD substrate with a short extension (Johnson et al., 1992), or due to the presence of specific residues that stabilize the structure and hamper unfolding (Lee et al., 2001). Notably, during the revision of this manuscript, it was reported that introduction of stable structures within a proteasome substrate can turn an otherwise normal substrate into a potent inhibitor (Navon and Goldberg, 2001). An alternative possibility is that UBB⁺¹ interferes more dramatically with degradation of the Ub^{G76V}-GFP substrate because these proteins are both UFD substrates and may well be targets for the same ubiquitin ligase. Accordingly, the stabilized UBB⁺¹ may competitively inhibit the ubiquitination of Ub^{G76V}-GFP.

Our model deviates from an earlier presented model that proposed poor deubiquitination of UBB⁺¹ as a possible cause for inhibition of the ubiquitin/proteasome system. Although we show that UBB⁺¹ can indeed inhibit the proteasome in vivo, and that this inhibitory activity relies on ubiquitination of UBB⁺¹, in accordance with the in vitro data (Lam et al., 2000), our results warrant a reevaluation of some of the observations in this earlier study. In the light of our results it is not surprising that ubiquitinated UBB⁺¹ is less efficiently disassembled than free polyubiquitin trees by isoT, considering that this deubiquitination enzyme is highly specific for free polyubiquitin trees rather than ubiquitinated substrates (Wilkinson et al., 1995). It will be interesting to compare in a similar deubiquitination assay if UBB⁺¹ is also more refractory to deubiquitination when compared with an authentic UFD substrate. The length de-

pendence of the ubiquitin tree is another puzzling aspect. We confirmed that the bulk of UBB⁺¹ in cell lysates contains one, two, or at most three conjugated ubiquitin moieties, whereas in the in vitro assay, UBB⁺¹ with synthetically linked Lys⁴⁸ tetraubiquitin was used, which fulfill much better the minimal length requirement for inhibitory polyubiquitin (Thrower et al., 2000). However, the interaction between substrates simultaneously ubiquitinated at Lys²⁹ and Lys⁴⁸ and the proteasome is not well understood, and it is possible that with these unique trees UBB⁺¹ can interact with the proteasome while bearing only a limited number of ubiquitins.

The critical significance of the UFD nature of UBB⁺¹ is further emphasized by the finding that introduction of multiple UFD signals had a dramatic enhancing effect on its inhibitory activity. Contrary to what we had expected on the basis of previously reported data (Stack et al., 2000), addition of one or two uncleavable ubiquitin moieties resulted in further accumulation of UBB⁺¹ and a stronger inhibition of the ubiquitin/proteasome system. Thus, in line with the hypothesis that cells can cope only with a certain level of ubiquitinated UBB⁺¹, when this level is increased by accelerating targeting UBB⁺¹ starts to accumulate and further inhibits its own degradation. The inhibitory activity of UBB⁺¹ may then establish a destructive feedback loop, which may ultimately result in overall inhibition of the ubiquitin/proteasome system.

In conclusion, we have provided evidence that UBB⁺¹ acts as a potent inhibitor of the ubiquitin/proteasome system in neuronal cells, and we have uncovered some important features of its mechanism of action. It remains to be seen whether and under what conditions this impaired proteolysis contributes to the generation of the protein aggregates that characterize many UBB⁺¹-associated pathologies. Finally, of paramount importance will be the identification of factors that can override the inhibitory effect of UBB⁺¹.

Materials and methods

Plasmid construction

All UBB⁺¹ and ubiquitin open reading frames were expressed from a CMV promoter in the mammalian expression vectors pcDNA3 (Invitrogen), pBK-CMV (Stratagene), EGFP-N1, or pCMS-EGFP (CLONTECH Laboratories, Inc.). The FLAG-tagged ubiquitin construct, FLAGUb, was generated by PCR amplification of ubiquitin from UBB⁺¹ and subsequent in-frame ligation into a FLAG-containing vector. Construction of the modified UBB⁺¹ constructs Ub*-UBB⁺¹ and Ub₂-UBB⁺¹ was based on a UBB⁺¹ plasmid in which an *NheI* site was introduced in between the ubiquitin moiety and the +1 extension of UBB⁺¹ (this also introduced a D79S amino acid substitution, although that did not affect its inhibitory capacity). The UBB⁺¹(*NheI*) was digested with *NheI*, and PCR-amplified Ub^{G76V} was ligated between the ubiquitin moiety and +1 extension. This procedure was repeated once to generate the Ub₂-UBB⁺¹ construct. Lys to Arg substitutions in the different constructs were introduced by PCR amplification. nGFP was constructed by introducing the amino acids substitution Y67R in the chromophore of GFP using PCR amplification. FLAGUb^{G76V}-nGFP, FLAGUb-R-nGFP, and FLAG_{p53} were generated by insertion of a double stranded oligonucleotide encoding the FLAG epitope as described previously (Heessen et al., 2002).

Transfections and tissue culture

The human cervical epithelial carcinoma line HeLa and neuroblastoma cell line SH-SY5Y were cultured in Iscove's modified Eagle's medium and high-glucose Dulbecco's modified Eagle medium, respectively, supplemented with 10% fetal calf serum (Life Technologies), 10 U/ml penicillin,

and 10 $\mu\text{g/ml}$ streptomycin. HeLa and SH-SY5Y cells were transiently transfected with Lipofectamine (Life Technologies) and calcium phosphate method, respectively. Cells were analyzed 48 h posttransfection unless stated otherwise. Stably transfected cell lines were selected in the presence of 0.5 mg/ml geneticin (Sigma-Aldrich) and screened for GFP fluorescence upon administration of proteasome inhibitors. Where indicated transfected cells were treated the reversible proteasome inhibitor MG132 (Affinity) or the irreversible proteasome inhibitors lactacystin, epoxomicin (Affinity) or Z-L₃-VS, a gift from Dr. Hidde Ploegh (Harvard Medical School, Boston, MA) (Bogyo et al., 1997)

Western blot analysis

Cell lysates were fractionated on SDS-PAGE and transferred to Protan BA 85 nitrocellulose filters (Schleicher & Schuell). The filters were blocked in PBS supplemented with 5% skim milk and 0.1% Tween-20, were and incubated with rabbit polyclonal antibody specific to UBB⁺ (Ubi-3, 050897; van Leeuwen et al., 1998), ubiquitin (Dako), or GFP (Molecular Probes). After subsequent washings and incubation with peroxidase-conjugated goat anti-rabbit serum, the blots were developed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). Quantification of Western blot bands was performed by densitometry (Molecular Dynamics).

Pulse-chase analysis

Neuroblastoma cells, SK-N-SH, were cultured and differentiated with retinoic acid. Differentiated SK-N-SH cells were transfected with a lentiviral-based vector (Naldini et al., 1996), containing the UBB⁺ open reading frame (lenti-UBB⁺). 24–48 h after transduction, cells were incubated in medium lacking methionine and cysteine for 1 h, and were subsequently metabolically labeled by incubating them with medium containing 100 μCi Tran35S-label for 4 h. After the labeling period, medium was replaced by Dulbecco's modified Eagle medium with 10% FCS medium. Cells were washed, chased with culture medium, and harvested at the indicated time points in 10 mM Tris, 0.15 M NaCl, 0.1% NP40, 0.1% Triton X-100, 20 mM EDTA, pH 8.0 buffer containing 0.1% SDS and protease inhibitors. UBB⁺ was immunoprecipitated overnight at 4°C with anti-UBB⁺ antibody Ubi-3 (1:1,000), and protein-A Sepharose beads were added to the UBB⁺ infected cell lysates. Analysis and quantification of the pulse-chase experiments were performed with the usage of a phosphoimager and the software package Imagequant software.

Fluorescence microscopy and flow cytometry

For fluorescence microscopy, the cells were grown and transfected on coverslips. After rinsing in PBS and fixation in 4% paraformaldehyde, immunostaining was performed using an anti-UBB⁺ rabbit polyclonal antibody or anti-FLAG mouse monoclonal antibody (M5; Sigma-Aldrich). After subsequent washing steps with PBS, cells were incubated with the secondary antibodies labeled Alexa Fluor 594 (Molecular Probes) or Texas red (Dako). All antibodies were diluted in 50 mM Tris, pH 7.4, 0.9% NaCl, 0.25% gelatine, and 0.5% Triton X-100. Cells were counterstained with Hoechst 33258 (Molecular Probes). Fluorescence was analyzed using a LEITZ-BMRB fluorescence microscope (Leica) and images were captured with a Hamamatsu cooled CCD camera. For quantitative analysis, 100–200 UBB⁺ or FLAG-Ub-positive cells per sample were scored for GFP fluorescence. Flow cytometry was performed with a FACSort flow cytometer (Becton Dickinson) and data were analyzed with CellQuest software. For analysis of cell cycle distribution, cells were harvested 2 d post transfection and fixed in 1% paraformaldehyde. After two washings in PBS, the cells were permeabilized with 70% ethanol and then incubated with propidium iodide. Flow cytometric analysis of the stability of Ub^{G76V}-GFP mutants was performed as described before (Dantuma et al., 2000a).

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