The effect of mutant ubiquitin on proteasome function in relation to neurodegenerative disease
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CHAPTER II

Dose-dependent inhibition of proteasome activity by a mutant ubiquitin associated with neurodegenerative disease

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Summary

The ubiquitin-proteasome system is the main regulated intracellular proteolytic pathway. Increasing evidence implicates impairment of this system in the pathogenesis of diseases with ubiquitin-positive pathology. A mutant ubiquitin, UBB⁺¹, accumulates in the pathological hallmarks of (i) tauopathies, including Alzheimer’s disease, (ii) polyglutamine diseases, (iii) liver disease and (iv) muscle disease and serves as an endogenous reporter for proteasomal dysfunction in these diseases. UBB⁺¹ is a substrate for proteasomal degradation, however it can also inhibit the proteasome. Here, we show that UBB⁺¹ properties shift from substrate to inhibitor in a dose-dependent manner in cell culture using an inducible UBB⁺¹ expression system. At low expression levels, UBB⁺¹ was efficiently degraded by the proteasome. At high levels, the proteasome failed to degrade UBB⁺¹, causing accumulation of UBB⁺¹ which subsequently induced a reversible functional impairment of the ubiquitin-proteasome system. Also in brain slice cultures, UBB⁺¹ accumulation and concomitant proteasome inhibition was only induced at high expression levels. Our findings show that by varying UBB⁺¹ expression levels, the dual proteasome substrate/inhibitory properties can be optimally employed to serve as research tool to study the ubiquitin-proteasome system and to further elucidate the role of aberrations of this pathway in disease.

Introduction

The main function of the ubiquitin-proteasome system (UPS) is proteolytic degradation of target substrates, including aberrant and misfolded proteins, to maintain cellular homeostasis (Glickman and Ciechanover, 2002). UPS-mediated post-translational regulation is also involved in many other cellular pathways such as transcription, DNA repair and endocytosis (Welchman et al., 2005). Ubiquitin (Ub) tags proteins for degradation through an enzymatic cascade, consisting of Ub activating (E1), Ub conjugating (E2) and Ub ligating (E3) enzymes. Via this pathway, a Ub is conjugated to internal lysine residues in substrate proteins. Through the sequential addition of Ub molecules to the substrate-bound Ub, a poly-Ub tree is formed which targets the substrate protein for degradation by the 26S proteasome (Glickman and Ciechanover, 2002; Pickart and Cohen, 2004).

As the UPS is important for maintaining intracellular homeostasis, it is not surprising that impairment of the UPS has been observed to occur in the pathogenesis of numerous diseases, often demonstrated by the accumulation of Ub conjugates or other components of the UPS machinery in protein aggregates (Ciechanover and Brundin, 2003). One of the disease-specific proteins which accumulates is ubiquitin-B⁺¹ (UBB⁺¹), a mutant form of Ub formed by a di-nucleotide deletion in the mRNA of the ubiquitin B gene (van Leeuwen et al., 1998). Previous in vitro results showed that UBB⁺¹ is ubiquitinated and appears
to be a protein with dual properties; on one hand it acts as a ubiquitin-fusion-degradation (UFD) substrate for the proteasome, on the other hand, it acts as a specific inhibitor of the proteasome (Lam et al., 2000; Lindsten et al., 2002). UBB1 accumulation eventually leads to apoptotic cell death (De Vrij et al., 2001; De Pril et al., 2004) and induces expression of heat-shock proteins (Hope et al., 2003).

In the diseased brain, UBB1 accumulates in the neuropathological hallmarks of tauopathies; e.g. in neuronal tangles in Alzheimer’s disease (AD), but also in astrocytes in progressive supranuclear palsy (Fischer et al., 2003; van Leeuwen et al., 2006). UBB1 is also found in intranuclear inclusions characteristic for polyglutamine diseases (De Pril et al., 2004). Outside the nervous system, UBB1 accumulates in the inclusion bodies of the muscle disease inclusion-body myositis (Fratta et al., 2004) and in Mallory bodies of chronic liver disease (McPhaul et al., 2002). We reported that UBB1 mRNA is present in equal levels in non-demented control individuals compared to AD patients (Fischer et al., 2003; Gerez et al., 2005). This suggests that the UBB1 mRNA transcript is always present. The UBB1 protein, however, seems to be efficiently degraded in healthy control subjects. Through a decline in UPS activity, for example by aging or disease related processes, the degradation of the UBB1 protein might be affected to such an extent that accumulation of the protein commences. Therefore we have proposed that UBB1 accumulation can serve as an endogenous reporter for a decreased UPS activity (Fischer et al., 2003). Once accumulated, UBB1 can contribute to disease pathogenesis by inhibiting the UPS (e.g. (Hol et al., 2005)). It is conceivable that this accumulation of UBB1 will only occur after exceeding a threshold level, causing a shift in the protein properties from proteasome substrate to proteasome inhibitor.

Indeed, results from the present study show that in human cell lines expressing inducible UBB1 levels, the UBB1 protein is degraded by the 26S proteasome at low expression levels and accumulates only after exceeding a threshold level of expression. In addition, we show that UBB1 exhibits dose-dependent UPS inhibitory properties. Our experiments show that UBB1 accumulation and subsequent UPS inhibition are both reversible processes. We further broadened the scope of these novel findings in cell lines to mouse organotypic cortex cultures, which closer resemble the human brain. In agreement with our in vitro results, UBB1 accumulation leads to successive inhibition of the UPS in these organotypic cultures only after surpassing a threshold level of expression. In conclusion, UBB1 is a protein which can be used as reporter for UPS activity as well as a highly selective dose-dependent UPS inhibitor by differentiating the levels of expression using (inducible) vectors with varying promoter constructs.
Materials and methods

Plasmid construction and viral constructs

Ub, UBB\(^{-1}\) and UBB\(^{-1}K29,48R\) open reading frames were cloned in pcDNA3 (Invitrogen) as described earlier (Lindsten et al., 2002). For the Tet-off inducible expression system the UBB\(^{-1}\) open reading frame was cloned downstream of the TRE-minimal cytomegalovirus immediate early (CMV) sequence of a Tet-off expression vector (pRevTRE; Clontech) into pcDNA3 and co-transfected with pRevTet-Off (Clontech). First generation recombinant adenoviral vectors Ad-UBB\(^{-1}\) and Ad-Ub were generated, purified and titered as described elsewhere (Hermens et al., 1997; De Vrij et al., 2001). Adenoviral vectors were based on the Ad5 mutant dl309 (Jones and Shenk, 1979) and employed the CMV promoter to drive transgene expression. Titration of double CsCl gradient-purified Ad-CMV-UBB\(^{-1}\) and Ad-CMV-Ub on the permissive cell line 911 (Fallaux et al., 1996) revealed titres of 1x10\(^9\) plaque forming units/ml. Lentiviral vectors were generated by cloning DNA encoding Ub-M-GFP, Ub\(^{G76V}\)-GFP, UBB\(^{-1}\) or UBB\(^{-1}K29,48R\) into the lentiviral transfer plasmid pRRLsin-ppThCMV. Lentivirus was produced according to Naldini et al. (Naldini et al., 1996) and harvested and titered as described previously using a HIV-1 p24 coat protein ELISA (NEN Research, Boston, USA) (De Pril et al., 2004). Virus titres were correlated to titres determined by counting GFP fluorescent cells of an LV-Ub-M-GFP stock. In this way titres of adenoviral and lentiviral stocks could be correlated.

Cell lines and transfections

The human cervical epithelial carcinoma cell line HeLa stably transfected with Ub\(^{G76V}\)-GFP (Dantuma et al., 2000) was cultured in high-glucose Dulbecco’s modified Eagle medium, containing 10% FCS, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (all Gibco). Stable cell line HeLa Ub\(^{G76V}\)-GFP was maintained on 60 μg/ml geneticin (G418; Gibco) selection. For Western blot and flow cytometry, HeLa Ub\(^{G76V}\)-GFP cells were plated on 6 or 12-wells plates with 1x10\(^5\) cells/well or 0.5x10\(^4\) cells/well respectively one day prior to transfection. Cells were transiently transfected with the calcium-phosphate method using 1 μg/ml DNA per vector. Where mentioned doxycycline (Sigma-Aldrich) treatment was applied 16 hours after transfection. Samples were harvested 48 hours after continuous doxycycline treatment, unless stated otherwise. Where indicated cells were additionally treated with the proteasome inhibitors MG132 (1 μM; Affiniti Research) or epoxomicin (100 nM; Affiniti Research) for 16 hours before samples were taken.
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Western blotting
For Western blotting, cells pellets were resuspended in suspension buffer (0.1 M NaCl, 0.01 M Tris-HCl pH7.6, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin) and lysed by 2 x 20 s sonification. Total protein content was quantified by Bradford measurement, equal protein amounts were fractionated by SDS-PAGE and blotted semi-dry to nitrocellulose filter (Schleicher and Schuell, Germany). UBB\(^{1}\) was detected using rabbit polyclonal anti-UBB\(^{1}\) antibody (Ub3 serum; 05/08/97; 1:1000 overnight (De Vrij et al., 2001)) and secondary swine anti-rabbit HRP (Dako; 1:1000) diluted in Supermix (0.05 M Tris-HCl, 0.9% NaCl, 0.25% gelatine and 0.5% Triton-X-100, pH7.4). Blots were developed by enhanced chemoluminescence (Lumilight ECL, Perkin Elmer, USA).

Flow cytometry
For flow cytometry, cell suspensions were fixed in 4% formalin in PBS, and resuspended in PBS-0.5% bovine serum albumin (Roche). GFP could be directly visualized, for UBB\(^{1}\) cytometry cells were stained with primary antibody anti-UBB\(^{1}\) (Ub3 serum; 1:500) and secondary antibody anti-rabbit Cy5 (Jackson ImmunoResearch; 1:400). Analysis was performed on at least 10000 cells per sample with a flow cytometer (FACSCalibur, Becton Dickinson Biosciences); data were analyzed using CellQuest software (Becton Dickinson Biosciences).

Organotypic cortex slice cultures
C57Bl/6 or C57Bl/6 Ub\(^{G76V}\)-GFP/2 tg mice (Lindsten et al., 2003) were decapitated at post-natal day 5; the brain was transferred to ice cold Gey’s Balanced Salt Solution (Sigma-Aldrich) containing 5.4 mg/ml glucose, 100 U/ml penicillin and 100 μg/ml streptomycin (all Gibco). After removal of the meninges, the fronto-parietal part was sliced into 300 μm coronal sections per hemisphere using a tissue chopper (McIlwain). The first four slices were discarded. Slices were cultured on an air-fluid interface on culture plate inserts (Millipore; 0.4 μm pore; 30 mm diameter; 3 cultures per insert) on medium containing 50% Minimum Essential Medium alpha, 25% HBSS, 25% horse serum, 6.5 mg/ml glucose, 2 mM glutamine (all Gibco) and penicillin/streptomycin (100 U/ml, 100 μg/ml). Viral transduction of cultures was achieved by applying 1x10\(^{6}\) transducing units of virus in a 10 μl droplet of culture medium on top of the slices. Treatment with epoxomicin (1 μM; Affiniti Research) or MG132 (10 μM; Affiniti Research) was performed in the same manner. Inhibitors were applied for 6 hours and subsequently left on or washed out overnight. Slices were stained free floating with rabbit polyclonal anti-UBB\(^{1}\) (Ub3 serum; 1:1000), rabbit polyclonal anti-GFAP (DAKO; 1:4000), monoclonal anti-GFP (Chemicon; 1:500) and monoclonal NeuN (Chemicon; 1:400) diluted in Supermix, followed by Cy2
and Cy3 staining (Jackson ImmunoResearch; 1:800) Nuclei were visualised with TO-PRO-3 (Molecular Probes; 1:1000). Subsequently, slices were mounted in mowiol (0.1 M Tris-HCl pH8.5, 25% glycerol, 10% w/v Mowiol 4-88) images were acquired using confocal laser scanning microscopy (Zeiss 510) and accompanying software (Zeiss LSM Image Browser).

Results

**UBB**\(^{+1}\) accumulates at high expression levels

We hypothesized that the previously described opposing properties of UBB\(^{+1}\) (Lindsten et al., 2002; Hol et al., 2005) can be explained by a dose-dependent shift from UPS substrate to inhibitor. In this study we quantified this effect in living cells using a HeLa cell-line stably expressing the Ub\(^{G76V}\)-GFP UPS reporter (Dantuma et al., 2000). Inducible levels of UBB\(^{+1}\) expression were achieved using the Tet-off gene-expression system. UBB\(^{+1}\) protein expression levels were analyzed with Western blot (Fig. 1) and Ub\(^{G76V}\)-GFP reporter accumulation was measured in the same sample using flow cytometry (Fig. 2). HeLa cells were transiently transfected with the UBB\(^{+1}\) Tet-off constructs and after 16 hours, doxycycline (dox) was added to the culture medium for 48 hours to regulate UBB\(^{+1}\) expression. Western blot analysis on cell lysates showed that UBB\(^{+1}\) protein accumulation was present only at high expression levels induced by absence of dox (maximal expression) or low dox concentrations ranging from 0 to 0.01 ng/ml (Fig. 1A). In addition, ubiquitination of UBB\(^{+1}\) (Ub-UBB\(^{+1}\)) was present in cells which showed UBB\(^{+1}\) accumulation and in cells transiently transfected with a CMV-UBB\(^{+1}\) pcDNA3 high expression control plasmid, as expected (Lindsten et al., 2002) (Fig. 1A). Ubiquitination of UBB\(^{+1}\) is essential for targeting UBB\(^{+1}\) to the proteasome. A double lysine mutant of UBB\(^{+1}\), UBB\(^{+1}\)K29,48R, cannot be ubiquitinated and is not targeted to the proteasome (Lindsten et al., 2002). Transient transfection with a CMV-UBB\(^{+1}\)K29,48R pcDNA3 plasmid indeed showed an increased accumulation of the non-ubiquitinated form of this protein compared to CMV-UBB\(^{+1}\) pcDNA3 (not shown).

Proteasomal degradation of UBB\(^{+1}\) could explain the absence of accumulation at lower expression levels. Therefore, we treated the UBB\(^{+1}\) Tet-off transfected cells with dox for 48 hours and simultaneously with the reversible proteasome inhibitor MG132 (1 μM) during the final 16 hours before sampling. Indeed, MG132 treatment shifted the regulated expression level at which UBB\(^{+1}\) accumulation could be observed to a lower level (Fig. 1B, lanes marked with arrowheads). From these experiments, we conclude that UBB\(^{+1}\) is degraded by the UPS at sub-maximal expression levels. However, the UPS fails to degrade UBB\(^{+1}\) sufficiently after exceeding a threshold level of expression, leading to stabilization of the UBB\(^{+1}\) protein.
Our previous work showed that UBB +1 can act as a proteasome inhibitor (Lindsten et al., 2002). Thus, we next determined the expression level at which inducible UBB +1 inhibited the proteasome and if this proteasome inhibition was dose-dependent. Therefore, we transfected stable HeLa UbG76V-GFP cells with the inducible Tet-off UBB +1 vectors as described in Fig 1. General inhibition of the proteasome leads to accumulation of GFP in this UbG76V-GFP HeLa cell line, a reporter cell line for UPS activity (Dantuma et al., 2000). Indeed, flow cytometric analysis of HeLa UbG76V-GFP cells treated overnight with proteasome inhibitors epoxomicin (irreversible inhibitor, 100nM) or MG132 (reversible inhibitor, 1 μM) showed a large increase in the percentage of GFP positive cells to ~90% (Fig. 2A). Transient transfection with CMV-UBB +1 pcDNA3 resulted in significant accumu-
mulation of the GFP reporter in ~10% of the living cells (Fig. 2A), reaffirming our previous observations that UBB\textsuperscript{+1} acts as a proteasome inhibitor (Lindsten et al., 2002). The discrepancy between the high levels of UPS inhibition achieved with classical inhibitors (~90% GFP positive) compared to UBB\textsuperscript{+1} (~10% GFP positive) is partially due to the transfection efficiency of UBB\textsuperscript{+1}, which is routinely ~40% in this cell line (data not
shown) and probably also due to the fact that not all UBB$^{+1}$ positive cells have built up enough expression to surpass the threshold level for UPS inhibition, corroborating our hypothesis that a critical level of UBB$^{+1}$ must be reached before the protein is stabilized. As expected, transient transfection with CMV-UBB$^{+1K29,48R}$ pcDNA3, which is not directed to the UPS, did not lead to significant UPS inhibition (Fig. 2A). Also transfection of wildtype Ub (CMV-Ub pcDNA3) did not lead to significant accumulation of the GFP reporter (Fig. 2A).

We determined if the proteasome inhibition induced by UBB$^{+1}$ was dose-dependent by transfecting HeLa Ub$^{G76V}$-GFP cells with the inducible Tet-off UBB$^{+1}$ vectors. Maximal UBB$^{+1}$ expression (absence of dox) was sufficient to induce UPS inhibition, shown by significant accumulation of the GFP reporter, although the percentage of GFP positive cells (4.4%) was lower than after transient transfection with CMV-UBB$^{+1}$ pcDNA3 (Fig. 2B). Furthermore, increasing GFP reporter accumulation was visible in a range from 0.01 to 0.0001 ng/ml dox, reaching significance at dox concentrations of 0.001 and 0.0001 ng/ml (Fig. 2B). This increase in the percentage of GFP positive cells and in the mean GFP fluorescence intensity is visualised in representative plots in Fig. 2C. These results indicate that UBB$^{+1}$ inhibited the proteasome in a dose-dependent manner, starting from expression levels at which UBB$^{+1}$ accumulation commenced.

**Accumulation of UBB$^{+1}$ and UPS inhibition is reversible in living cells**

The previous results showed that increasing UBB$^{+1}$ expression gave rise to dose-dependent UPS inhibition. It is conceivable that this UPS inhibition is irreversible, causing a defective degradation of previously accumulated UBB$^{+1}$ even after UBB$^{+1}$ expression is shut down. To verify this we measured the percentage of remaining UBB$^{+1}$ positive cells after shutting down UBB$^{+1}$ expression. Ub$^{G76V}$-GFP HeLa cells were transiently transfected with the Tet-off UBB$^{+1}$ vectors and expressed high levels of UBB$^{+1}$ for 64 hours, after which a baseline sample was taken (timepoint 0 hours). UBB$^{+1}$ expression was continued at high levels and additional samples were taken at 12 hours and 36 hours after the baseline measurement. Alternatively, UBB$^{+1}$ expression was shut down by adding 10 ng/ml dox and samples were taken at similar timepoints. The percentages of UBB$^{+1}$ (Fig. 3A) positive cells and GFP/UBB$^{+1}$ double positive cells indicative for UPS inhibition (Fig. 3B) were determined for every timepoint using flow cytometry (minimal 10000 cells counted per sample).

At the baseline measurement (0 hours), maximal UBB$^{+1}$ levels were present resulting in 5.3% UBB$^{+1}$ positive cells (Fig. 3A) leading to 1.3% UBB$^{+1}$/GFP double positive cells (Fig. 3B). Thus ~25% of the UBB$^{+1}$ positive cell population also accumulated the GFP reporter, indicating that UBB$^{+1}$ accumulation preceded inhibition of the UPS, which is in agreement with earlier observations in a different setup (Lindsten et al., 2002). After 12
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UBB+1 threshold

hours, continuous UBB+1 expression (dox-) or shutting down expression (dox+) both gave rise to equal slight decreases in UBB+1 protein levels. However, constant UBB+1 expression (dox-) induced a ~50% decrease in the number of UBB+1 positive cells from 3.8% to 1.9% at 36 hours compared to 12 hours after baseline measurement (Fig. 3A). This is presumably caused by either loss of the expression plasmid after transient transfection, or by loss of UBB+1 due to UBB+1 induced cell death as seen in previous experiments (De Vrij et al., 2001). In the condition where the expression of UBB+1 was shut down (dox+) for 36 hours, the percentage of UBB+1 positive cells dropped with ~75% compared with levels 12 hours after dox treatment from 4.2% to 1.0%. This remaining percentage of UBB+1 positive cells (1.0%) was significantly lower than when the cells had continuous UBB+1 expression (1.9%). This indicated that UBB+1 accumulation was reversible at 36 hours after shutting down expression, although the remaining UBB+1 accumulation stayed significantly elevated compared to a transfection control sample (0.3%) (Fig. 3A).

The percentage of UBB+1/GFP double positive cells remained stable after 12 hours in both the dox treated and untreated condition (Fig. 3B). After 36 hours, this population of cells decreased in both the continuous UBB+1 expression condition (dox-) and after shutting down expression (dox+). However, similar to the results for UBB+1 accumulation, the UBB+1/GFP positive cell population was lower in the dox treated condition (dox+) than in the condition with constant UBB+1 expression (dox-), although this effect was not significant (Fig. 3B). This lower amount of GFP reporter accumulation in the condition where

Figure 3 Accumulation of UBB+1 is reversible after shutting off expression. Flow cytometric analysis of the percentage of UBB+1 (A) or UBB+1/GFP (B) positive cells at 0 hours, 12 hours and 36 hours after shutting down maximal UBB+1 expression. UbG76V-GFP HeLa cells were not transfected (control), transfected with empty pcDNA3 vector (e) or transfected with the UBB+1 Tet-off vectors. After 64 hours of maximal expression (absence of dox) a baseline sample was taken (0 hours). UBB+1 expression was continued at maximal levels (dox-) or shut down by addition of 10 ng/ml dox (dox+) and samples were analyzed after 12 hours and 36 hours of dox treatment. Results are the means ± s.e.m. of two or three independent duplicate experiments. * p<0.01, # p=0.099 ANOVA within timegroup.
UBB⁺¹ expression has been shut down for 36 hours indicated that the UPS inhibition induced by UBB⁺¹ was a reversible process. Moreover, possible UBB⁺¹ induced cell death should be lower when UBB⁺¹ expression is shut down, so the decrease in GFP accumulation can then only be attributed to the reversal of UPS inhibition.

To corroborate these data, we also used a experimental setup where high levels of UBB⁺¹ expression were induced with lentiviral (LV) vectors in a human neuroblastoma cell line (SH-SY5Y). By using LV transduction, the UBB⁺¹ cDNA integrated in the cellular DNA, leading to stable expression of the protein. With this high continuous LV-UBB⁺¹ expression, experiments could be done in a shorter time span in which UBB⁺¹ induced cell death did not occur yet. In this setup, LV-UBB⁺¹ transduction induced UBB⁺¹ accumulation which further increased after overnight inhibition of the UPS with the reversible inhibitor MG132. After restoring proteasome activity by removing MG132, the percentage of UBB⁺¹ positive cells decreased significantly compared to the condition in which MG132 was not removed (data not shown), indicating that UBB⁺¹ accumulation is a reversible process. The percentage of UBB⁺¹ positive cells in the condition with maximal UBB⁺¹ expression was comparable to the percentage of positive cells after a control LV-UBB⁺¹K29,48R infection. It is known from previous experiments that this control LV-
UBB+1 THRESHOLD

Degradation of UPS substrates in organotypic cortex cultures

We then investigated if these results in cell lines also hold true in primary (neuronal) cultures using mouse organotypic cortex slice cultures, so that we could extrapolate our data to neurodegenerative disease conditions. First, we verified that transduction of cells was possible in this setup using LV vectors. Cortex slice cultures of C57Bl/6 mice were infected with Ub-M-GFP, a ubiquitin fusion protein that results in a stable form of GFP (Dantuma et al., 2000). This gave rise to many GFP immuno-positive cells two days after transduction (Fig. 4). The transduced cell population consisted mainly of astrocytes, although also a substantial amount of GFP positive neurons was present in the slice, as demonstrated by double-staining with neuronal (NeuN) and glial (glial fibrillary acidic protein (GFAP)) markers (Fig. 4).

To assess general UPS function in the cortex culture system, slices were LV transduced with the UPS reporter construct UbG76V-GFP. As shown in Figure 5, UbG76V-GFP was efficiently degraded by the proteasome and GFP accumulation was only present after

Figure 5  UBB+1 is degraded by the proteasome in cortex slice cultures. Organotypic cortex slices of C57Bl/6 mice were transduced with LV-UbG76V-GFP, LV-UBB+1 or LV-UBB+1K29,48R. Both the UPS reporter protein UbG76V-GFP (green) and UBB+1 (red) are efficiently degraded by the 26S proteasome and only accumulate after treatment with proteasome inhibitor. The lysine mutant of UBB+1, UBB+1K29,48R, is not degraded by the proteasome an accumulates already without inhibitor treatment; - epox: not treated with epoxomicin, + epox: treated overnight with 1 μM epoxomicin. Bar, 100 μm. See color section.

UBB+1K29,48R construct does not induce cell death (De Pril et al., 2004), thereby ruling out the possibility that UBB+1 induced cell death affected the reversibility of UBB+1 accumulation in this setup.
treatment with proteasome inhibitor (1 μM epoxomicin) (Fig. 5). Transduction of cortex cultures with LV-UBB+1 also did not lead to accumulation of the protein (Fig. 5), in contrast to the neuronal cell cultures. Apparently, the UBB+1 protein was efficiently degraded by the 26S proteasome in cortex slice cultures, as demonstrated by the accumulation of UBB+1 after overnight proteasome inhibition by 1 μM epoxomicin (Fig. 5). These results indicate that LV-UBB+1 expression leads to sub-threshold expression in this cortex slice system at which the protein does not accumulate. The LV-UBB+1K29,48R mutant showed accumulation of the protein regardless of proteasome inhibition, as expected (Fig. 5).

**UBB+1 accumulation is not reversible in cortex cultures**

We made use of the reversible proteasome inhibitor MG132 to study if accumulation of UBB+1 in cortex cultures also decreased after wash out of the proteasome inhibitor as we observed in the neuroblastoma cell line experiments. Consistent with results obtained with epoxomicin treatment as shown above, applying MG132 overnight to LV transduced cultures resulted in strong accumulation of both UbG76V-GFP and UBB+1 (Fig. 6). When MG132 treated cultures were rinsed and allowed to recover, proteasome activity was restored, as demonstrated by the regained capacity to completely degrade the GFP reporter.

![Figure 6](image)

*Figure 6  UBB+1 remains present after washout of inhibitor in cortex slice cultures. Overnight incubation of cortex cultures transduced with LV-UbG76V-GFP or LV-UBB+1 with the reversible proteasome inhibitor MG132 (10 μM) resulted in accumulation of both proteins. Washing out the reversible inhibitor reactivated the proteasome, as shown by the degradation of the proteasome reporter substrate UbG76V-GFP. However, UBB+1 remained accumulated in a considerable amount of cells after reactivation of the proteasome. Transduction with the LV-UBB+1K29,48R control construct gave rise to accumulation of the UBB+1 protein regardless of proteasome inhibitor treatment. UbG76V-GFP is depicted in green, UBB+1 in red and the nuclear staining (TO-PRO) in blue. Bar, 500 μm. See color section.*
protein (Fig. 6). This restored proteasome activity did not seem capable of degrading accumulated UBB\(^+1\) under these washout conditions, as the number of cells containing accumulated UBB\(^+1\) after washout of the inhibitor was similar to the number of UBB\(^+1\) positive cells after initial transduction (Fig. 6). This indicated that, in contrast the results obtained in cell lines, UBB\(^+1\) accumulation in cortex cultures remained present after washout of the proteasome inhibitor, which might be due to the recovery time needed after washout to degrade UBB\(^+1\).

**Accumulated UBB\(^+1\) inhibits the UPS in cortex cultures**

*In vitro*, UBB\(^+1\) inhibits the UPS after exceeding a threshold as shown in a Ub\(^{G76V}\)-GFP stable HeLa cell line. We employed a Ub\(^{G76V}\)-GFP transgenic (tg) mouse line (Ub\(^{G76V}\)-GFP/2) to translate these results to our cortex culture setup. In this mouse model, the UPS reporter is ubiquitously expressed and similar to the HeLa cell line, GFP accumulates only after proteasome inhibition (Lindsten *et al.*, 2003). To verify the GFP proteasome reporter system in organotypic cortex slices of Ub\(^{G76V}\)-GFP/2 tg mice, the cortex slices were cultured and treated with 1 \(\mu\)M epoxomicin. Indeed, the GFP reporter only accumulated in the cortex slice cultures after treatment with proteasome inhibitor (Fig. 7). Similar to cortex slices from non-tg mice, in the Ub\(^{G76V}\)-GFP/2 cortex slices, LV-UBB\(^+1\) transduction did not lead to UBB\(^+1\) accumulation (Fig. 8B) unless additional proteasome inhibitors were applied (not shown). This additional proteasome inhibitor treatment led to GFP reporter accumulation in the tg cultures regardless of UBB\(^+1\) expression, making it impossible to distinguish proteasome inhibition by the inhibitor or by UBB\(^+1\). Therefore, we used adenoviral (Ad) instead of LV transduction of UBB\(^+1\) to induce higher expression levels, which might exceed the accumulation threshold. We confirmed the increased expression...
of Ad-UBB⁺¹ (right lane, Fig. 8A) compared to LV-UBB⁺¹ (left lane, Fig. 8A) in HEK293 cells, showing that Ad-UBB⁺¹ transduction resulted in 4- to 5-fold higher expression of UBB⁺¹ compared to LV transduction (Fig. 8A). Transduction of Ub₇⁶V-GFP tg cortex cultures with Ad-UBB⁺¹ indeed resulted in accumulation of UBB⁺¹ in many cells (Fig. 8C), in contrast to LV-UBB⁺¹ transduction (Fig. 8B). The majority of cells that were positive for UBB⁺¹ after Ad-UBB⁺¹ transduction clearly accumulated the GFP reporter (Fig. 8D). UBB⁺¹ accumulation was observed mainly in the cytosol, whereas the GFP reporter accumulation was present in the cytosol as well as in the nucleus. Similar to the in vitro results, inducing a high level of UBB⁺¹ expression in organotypic cortex slice resulted in surpassing the accumulation threshold and subsequent inhibition of the proteasome.

Discussion

Our previous results indicated that UBB⁺¹ behaves both as a substrate as well as an inhibitor of the UPS (Lindsten et al., 2002; Fischer et al., 2003). In this study we further explored the dual UPS substrate/UPS inhibitor properties of UBB⁺¹, using novel tet-off inducible UBB⁺¹ expression vectors vary levels of UBB⁺¹ expression. We show here that a concentration-dependent shift in UBB⁺¹ properties from UPS substrate to inhibitor takes place with increasing expression levels. UBB⁺¹ accumulation commenced only at high expression levels and preceded the induction of UPS inhibition. In addition, we show in this study that both UBB⁺¹ accumulation and UPS inhibition were partially reversible after
ceasing UBB\textsuperscript{+1} expression. We further studied UBB\textsuperscript{+1} characteristics in organotypic cortex slice cultures, a system which reflects a multi-cellular environment in which neuronal connectivity and neuron-glia interactions are preserved (Sundstrom et al., 2005). This study is, to our knowledge, the first employing organotypic cultures to assess UBB\textsuperscript{+1} properties. In these cultures, UBB\textsuperscript{+1} accumulation and subsequent UPS inhibition only occurred at high levels of expression, similar to the results obtained in cell lines. In summary, the current study shows that UBB\textsuperscript{+1} properties dose-dependently shift from a proteasome substrate to a partially reversible proteasome inhibitor after a critical level of accumulation is reached.

We hypothesized that UBB\textsuperscript{+1} might be able to irreversibly sustain or even increase its own accumulation through a “feedback loop” of UBB\textsuperscript{+1}-induced UPS inhibition. Surprisingly, a clear decrease was observed in the percentage of UBB\textsuperscript{+1} positive cells after shutting down expression of UBB\textsuperscript{+1} (Fig. 3A) or recovery after UPS inhibition, although UBB\textsuperscript{+1} levels remained elevated compared to the levels before treatment. It is conceivable that this decline of UBB\textsuperscript{+1} accumulation continues over time, clearing the remaining accumulation after a longer period of recovery. This reversible accumulation was not as clear in cortex slices; many UBB\textsuperscript{+1} positive cells remained present after recovery of proteasomal inhibition in a setup where full degradation of the UPS reporter Ub\textsuperscript{G76V}-GFP was observed (Fig. 6). In these slices, 16 hours of UPS recovery might not be sufficient to observe a clear UBB\textsuperscript{+1} degradation. These results indicate that the UBB\textsuperscript{+1} protein likely has a longer half-life than Ub\textsuperscript{G76V}-GFP in cortex slices, corresponding to previous cell line observations regarding the half-life of UBB\textsuperscript{+1} compared to Ub\textsuperscript{G76V-GFP} (Lindsten et al., 2002). Also, a slight decrease in UBB\textsuperscript{+1} intensity or number of UBB\textsuperscript{+1} positive cells might not be detectable in cortex slices as an exact quantification is not feasible due to variation. Alternatively, primary neurons and astrocytes might respond differently to the inhibition of the UPS compared to tumour cell lines.

A point of interest is that LV vectors are known to efficiently transduce neuronal cells in culture (Ehrengruber et al., 2001). In our organotypic slices we observed that the majority of the infected population consisted of GFAP positive glial cells, although neuronal cells were also transduced (Fig. 4). In this respect it is important to note that in the human brain, UBB\textsuperscript{+1} not only accumulates in neurons in for instance AD, but also in glial cells of white matter in e.g. progressive supranuclear palsy (Fischer et al., 2003). The UBB\textsuperscript{+1} protein was mainly localized in the cytosol of the transfected cells in the slices. Surprisingly, UBB\textsuperscript{+1} positive cells showed accumulation of the Ub\textsuperscript{G76V}-GFP reporter in both the cytosol and in the nucleus (Fig. 8D). Intranuclear localisation of the GFP reporter was also seen in Ub\textsuperscript{G76V}-GFP tg cortex cultures (Fig. 4) and in neuronal cultures from a comparable Ub\textsuperscript{G76V}-GFP tg line (Lindsten et al., 2003) treated solely with proteasome inhibitor, indicating that general UPS inhibition results in both cytosolic and nuclear accumulation of the GFP-reporter.
Besides the UBB\textsuperscript{1} protein, shown in this study to be a dose-dependent reversible UPS inhibitor, many other compounds are known that inhibit the proteolytic activity of the 26S proteasome. These inhibitors can be divided into several major classes such as the synthetic peptide aldehydes, which act upon reversible binding (e.g. MG132, MG115 and PSI), the peptide boronates, a class of highly selective reversible inhibitors which have a slow on-off rate (e.g. MG262 and PS-341) and peptide vinyl-sulfones which bind irreversibly to the 20S core. Lactacystin and the specific 26S proteasome inhibitor epoxomicin are natural non-peptide irreversible proteasome inhibitors (reviewed in (Kisselev and Goldberg, 2001; Myung \textit{et al.}, 2001)). In this respect, UBB\textsuperscript{1} can be classified as an endogenously encoded inhibitor of the UPS, which induces dose-dependent reversible UPS inhibition (Fig. 1B). UPS inhibition by UBB\textsuperscript{1} is a specific effect of UBB\textsuperscript{1} and not due to overloading the UPS by over-expressing a UPS substrate, as comparable expression of other UPS substrates such as $^{\text{FLAG}}\text{Ub}^{\text{G76V}}$-nfGFP, $^{\text{FLAG}}\text{Ub}$-R-nfGFP or $^{\text{FLAG}}$p53 did not inhibit the UPS (Lindsten \textit{et al.}, 2002). Through the possibility to vary UBB\textsuperscript{1} expression levels in different models systems (e.g. cell lines, primary cultures and transgenic animal models) using inducible vectors, the dual UPS substrate and UPS inhibitory properties of this protein can be optimally employed.

In this study we further validate our hypothesis that the presence of UBB\textsuperscript{1} serves as a marker for proteasomal dysfunction in human neurodegenerative disease (Fischer \textit{et al.}, 2003; Hol \textit{et al.}, 2005), showing in human cell lines and neuronal slice cultures that inhibition of the proteasome using proteasome inhibitors can induce the accumulation of UBB\textsuperscript{1}, even if at these specific expression levels UBB\textsuperscript{1} is normally degraded. Accordingly, UBB\textsuperscript{1} accumulation in human pathology can serve as an endogenous marker for UPS inhibition, which holds UPS inhibitory properties itself (Fig. 9). Recently, it was shown that a decline in proteasome activity induced by classical proteasome inhibitors impaired protein synthesis in neuronal cells (Ding \textit{et al.}, 2006) and disrupted multiple processes in ribosome biogenesis (Stavreva \textit{et al.}, 2006). After washout of the reversible proteasome inhibitor MG262, levels of protein synthesis were restored (Ding \textit{et al.}, 2006). As UBB\textsuperscript{1} is also an inhibitor of the proteasome, it is possible that we underestimated the extent of UBB\textsuperscript{1} accumulation in our experiments due to a decreased UBB\textsuperscript{1} synthesis. Also the protein synthesis levels of the GFP-reporter might be decreased. Conversely, the reversal of UBB\textsuperscript{1} accumulation after shutting down UBB\textsuperscript{1} expression might be influenced by an increased level of protein synthesis, possibly slowing the process of accumulation reversal. Decreases in ribosome function and protein synthesis are also associated with aging (reviewed by (Rattan, 1996)) and neurodegenerative disease (Ding \textit{et al.}, 2005). Possibly, the accumulated UBB\textsuperscript{1} in the neurodegenerative disease hallmarks further contributes to these processes via its UPS inhibitory properties. Future experiments could further clarify this issue.
The reversible inhibitory properties of UBB\(^{+1}\) can prove to be useful in many research fields besides neurobiology. The UPS is most well-known is its function in protein quality control, but increasing significance is attributed to its role in development, endocytosis, DNA repair and transcriptional regulation (Welchman et al., 2005). In this respect, the role of UPS regulated protein turnover in cell cycle progression has become of major importance in cancer research, as oncogenic mutations can be found which perturb ubiquitination of cell cycle proteins and induce the disruption of intracellular balance between cell growth and death characteristic for cancer cells (Mani and Gelmann, 2005). Therefore it is not surprising that proteasome inhibitors have emerged as attractive drug targets for

**Figure 9** UBB\(^{+1}\) properties shift from UPS substrate to inhibitor. (1) UBB\(^{+1}\) mRNA and translation levels are constant throughout life (Fischer et al., 2003; Gerez et al., 2005). In non-diseased tissue, the 26S proteasome is capable of degrading all the translated UBB\(^{+1}\) and accumulation of UBB\(^{+1}\) is not present. (2) Due to various causes such as disease or aging the efficiency of proteasomal degradation can decrease, leading to a diminished degradation of UBB\(^{+1}\). (3) The levels of translated UBB\(^{+1}\) exceed the degradation capacity of the proteasome and surpasses the accumulation threshold. Accumulated UBB\(^{+1}\) now holds UPS inhibitory properties itself, which can aggravate the initial decrease in UPS activity. See color section.
e.g. cancer therapy (Hol et al., 2006; Nalepa et al., 2006). The first clinically approved anti-cancer drug in this respect is bortezomib (Velcade®), a small-molecule UPS inhibitor used to treat malignant multiple myeloma (reviewed in (Rajkumar et al., 2005)), which can induce apoptosis in tumour cells (Adams, 2004). As UBB+1 is an endogenously encoded UPS inhibitor which induces apoptosis, it is possible to mediate tissue-specific gene delivery by viral vectors. This unique combination makes it worthwhile to further explore the potential of UBB+1 as a tissue specific UPS inhibitor in disease.

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