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Mutant ubiquitin expressed in Alzheimer’s disease causes neuronal death

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ABSTRACT

Ubiquitin-B\textsuperscript{+1} (UBB\textsuperscript{+1}) is a mutant ubiquitin that accumulates in the neurons of patients with Alzheimer's disease (AD). Here we report on the biochemical and functional differences between ubiquitin and UBB\textsuperscript{+1} and the effect of the mutant protein on neuronal cells. UBB\textsuperscript{+1} lacks the capacity to ubiquitinate, and although it is ubiquitinated itself, UBB\textsuperscript{+1} is not degraded by the ubiquitin-proteasomal system and is quite stable in neuronal cells. Overexpression of UBB\textsuperscript{+1} in neuroblastoma cells significantly induces nuclear fragmentation and cell death. Our results demonstrate that accumulation of UBB\textsuperscript{+1} in neurons is detrimental and may contribute to neuronal dysfunction in AD patients.
INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia, affecting a large group of the elderly. Many genetic and nongenetic factors have been implicated in the pathogenesis of AD (Cummings et al., 1998b). We recently demonstrated that dinucleotide deletions in mRNA occur in AD brains, resulting in translation and accumulation of frameshifted so-called +1 proteins which may affect neuronal functioning. The process by which these dinucleotide deletions form is termed molecular misreading (Van Leeuwen et al., 1998b). Several examples of these aberrant +1 proteins have been found in the hallmarks of AD. Molecular misreading of the ubiquitin B gene results in UBB+1 protein, which accumulates in the earliest affected brain areas of patients with AD (Braak and Braak, 1991) (e.g., pyramidal cells in the CA1 region of the hippocampus, and neurons in the subiculum and cortex of AD patients) (Van Leeuwen et al., 1998a). Recently, Lam et al. (Lam et al., 2000) and we (Van Leeuwen et al., 1998a; Van Leeuwen et al., 2000) proposed that UBB+1 in the diseased aging brain could result in a dominant negative inhibition of the major proteolysis system, the ubiquitin-proteasomal system (Ciechanover, 1998). This blockade of protein degradation might contribute to the process of neuropathology in AD.

The ubiquitin-proteasomal system is involved in many cellular processes, such as cell cycle, apoptosis, endocytosis and ATP-dependent proteasomal breakdown of proteins (Ciechanover, 1998). Ubiquitin (Ub) tags proteins for degradation by conjugating to substrates through isopeptide bonds between the carboxy-terminal glycine residue of Ub and the ε-amino groups of lysine residues in proteins. Branched multi-Ub chains are formed by the sequential addition of mono-Ub to a lysine residue of substrate-bound Ub (Varshavsky, 1997). Such a multi-Ub chain serves as a targeting signal, resulting in degradation of the protein by the 26S proteasome (Ciechanover, 1998).

UBB+1 is a putative target for ubiquitination by wild-type Ub as it still contains the lysine residue at position 48. On the other hand, the UBB+1 protein is probably not able to participate actively in forming multi-ubiquitin trees itself, as it lacks the carboxyl-terminal glycin moiety. Recently, ubiquitinated UBB+1 has been found to inhibit the proteasome in a cell-free system (Lam et al., 2000). Inhibition of the ubiquitination process or a dominant negative effect on proteasomal
breakdown is likely to cause problems in protein degradation and therefore in neuronal functioning.

Here, we report on the biochemical properties of UBB\(^{+1}\) in an \textit{in vitro} system and on the differential effects of wild-type Ub and UBB\(^{+1}\) on cellular functioning achieved by overexpressing these proteins in the human neuroblastoma cell line SK-N-SH. Our data show that UBB\(^{+1}\) was ubiquitinated but, in contrast to other ubiquitinated proteins, was not degraded. Moreover, we show that UBB\(^{+1}\) changed cell morphology, followed by neuronal cell death.

\section*{MATERIALS AND METHODS}

\subsection*{\textit{In vitro} experiments with UBB\(^{+1}\)}

Rabbit reticulocyte lysates were used as the source of enzymes participating in the Ub degradative pathway. ATP- and ubiquitin-depleted fraction II was prepared from rabbit reticulocyte lysates as described by Hershko et al. (Hershko et al., 1982).

Ubiquitin conjugation experiments (Fig. 1) were performed according to Gregori et al. (Gregori et al., 1995). Recombinant His-tagged UBB\(^{+1}\), purified on a Ni\(^{+}\) column and ubiquitin wild-type (wtUb) were iodinated using the iodogen method (Pierce, Rockford, IL). The specific activities were \(3 \times 10^5\) cpm/\(\mu g\) and \(6.4 \times 10^5\) cpm/\(\mu g\) for UBB\(^{+1}\) and wtUb, respectively. Approximately 1 \(\mu\)M of wtUb or UBB\(^{+1}\) was used in the reaction mixture in the presence or absence of ATP. At the indicated times, an aliquot of the reaction mixture was removed, the reaction was stopped by the addition of gel electrophoresis sample buffer containing 1\% SDS and 0.5\% \(\beta\)-mercaptoethanol, boiled for 3 minutes, and subjected to gel electrophoresis. The gel was dried and ubiquitin conjugates were visualized by autoradiography.

The conditions for ubiquitin and ATP-dependent degradation of iodinated substrates have been described elsewhere (Gregori et al., 1995). Lysozyme was used as the control for the degradation reaction. Lysozyme-specific radioactivity was \(10^7\) cpm/\(\mu g\). Unlabeled
ubiquitin (10 μM) was added to the reaction mixture in addition to iodinated UBB\(^{+1}\) and wtUb, both at the final concentration of 1 μM (Fig. 2). Iodinated lysozyme concentration was 15 μM. During degradation, digested proteins were reduced to amino acids and small peptides that are acid-soluble. Radioactivity in the acid-soluble fraction was measured with a gamma counter and reported as the percentage of total radioactivity. The results shown in Fig. 2 represent the average values and the SE of the mean of a triplicate experiment.

**Cell lines**

SK-N-SH neuroblastoma cells were cultured in high-glucose Dulbecco's modified Eagle medium (Life Technologies, Grand Island, NY) containing 10% foetal calf serum (FCS) (Life Technologies) and supplemented with 100 U/ml penicillin (Life Technologies) and 100 μg/ml streptomycin (Life Technologies) (DMEM-10% FCS). Cells were cultured on poly-L-ornithine-hydrobromide (Sigma, St. Louis, MO)-coated glass coverslips in 24-wells plates (Nunc, Roskilde, Denmark) 1 day prior to infection. In one experiment 0.2% gelatin coating was used on the glass coverslips, which gave similar results.

*Fig. 1 UBB\(^{+1}\) is not able to conjugate proteins.* Autoradiograph of iodinated wtUb (left panel) and iodinated UBB\(^{+1}\) (right panel) incubated with fraction II of rabbit reticulocyte lysate in the presence (+) or absence (-) of ATP at different times (indicated in minutes). It is clear that in the presence of ATP, only wtUb is able to conjugate proteins. Arrows indicate monomeric wtUb and UBB\(^{+1}\).
Viral constructs

First generation recombinant adenoviral vectors were constructed, purified and titered as described elsewhere (Hermens et al., 1997). All three viral vectors (AdLacZ, AdUBB\(^{+1}\) and AdwtUb) are based on the Ad5 mutant \textit{dl309} (Jones and Shenk, 1979) and use the cytomegalovirus immediate early (CMV) promoter to drive transgene expression.

Titration of double CsCl gradient-purified Ad-CMV-LacZ, Ad-CMV-UBB\(^{+1}\) and Ad-CMV-wtUb on the permissive cell line 911 (Fallaux et al., 1996) revealed titres of 1 x 10\(^5\), 4 x 10\(^5\) and 1 x 10\(^6\) plaque forming units/ml, respectively. The presence of replication of competent adenovirus (RCA) in the adenoviral UBB\(^{+1}\) stock was determined by standard procedure titration on A549 cells (Hermens et al., 1997). No cytopathic effect in relation to RCA was observed.

Infections of neuroblastoma cells

One day prior to infection, neuroblastoma cells were cultured in 24-wells plates (2 x 10\(^5\) cells/well). The next day the cells were differentiated by adding medium with 4 \(\mu\)M all-trans retinoic acid (Sigma) (Slack et al., 1992). Cells were infected 4 h later with a multiplicity of infection (MOI) of 10 with Ad-CMV-wtUb, Ad-CMV-UBB\(^{+1}\), or Ad-CMV-LacZ as a control. Medium containing the adenoviral vectors was left on for 2 h, after which medium was replenished with normal medium containing retinoic acid. Cells were fixed at different times after infection (1, 3, and 6 days).

Western blots

Pellets of neuroblastoma cells were resuspended in suspension buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) containing protease inhibitors PMSF and leupeptin, in concentrations of 100 \(\mu\)M and 10 \(\mu\)g/ml respectively, or in SDS loading buffer containing 1mM NEM (Sigma).

All samples were loaded onto 15% SDS-PAGE gels and then transferred semi-dry onto nitro-cellulose. Blots were then probed with rabbit antisera to wtUb (anti-wtUb, 1:500, DAKO, Carpinteria, CA) or UBB\(^{+1}\) (Ubi3\(^{+1}\) serum, 05/08/97, 1:1000, epitope: YADLREDPDRQ).
Subsequently, blots were incubated with anti-rabbit HRP (DAKO, 1:1000) and Lumilight ECL (Boehringer, Mannheim, Germany) chemiluminescence.

**Pulse chase assay**

SK-N-SH cells were cultured in 3.5cm dishes (Nunc) and differentiated with retinoic acid when confluency was reached. Cells were infected with the different Ad vectors as described previously. One day after infection, cells were incubated in medium lacking methionine and cysteine for 1 h, after which the cells were labeled by incubating them with medium containing 100 μCi Tran35S-label for 4 h at 37°C and 5% CO₂. After labeling, the medium was replaced by DMEM-10% FCS medium. Cells were harvested at different times in buffer (10 mM Tris, 0.15 M NaCl, 0.1% Nonidet P40, 0.1% Triton-x-100, 20 mM EDTA, pH 8.0) containing 0.1% SDS and protease inhibitors. For immunoprecipitation, anti-UBB⁺⁻ antibody Ubi3⁺⁻ (1:1000) and protein-A Sepharose beads were added to the Ad-UBB⁺⁻-infected cell lysates. This suspension was shaken overnight at 4°C. The next day, beads were spun down and the supernatant was replaced with buffer containing 0.1% SDS. The pellet was rinsed three times in buffer and twice in 10 mM Tris-HCl, pH 8.0. Subsequently, loading buffer was added to the Sepharose beads, after which the samples were boiled and loaded on a 15% SDS-PAGE gel. Gels were visualized using a PhosphorImager.

**Immunofluorescence**

After infection, cells were fixed in 4% formalin in phosphate buffered saline (PBS), pH 7.4, for 1 h or longer. In between staining steps, cells were rinsed with PBS, pH 7.4. Rabbit polyclonal anti-UBB⁺⁻ (Ubi3⁺⁻ serum, 05/08/97), rabbit polyclonal anti-wtUб (Sigma) and monoclonal anti-β-gal (gal-13, Sigma) antibodies were diluted 1:500, 1:100 and 1:350, respectively. The secondary anti-rabbit-Cy3 and anti-mouse-Cy3 were diluted 1:200 in Supermix containing 0.05M Tris, 0.9% NaCl, 0.25% gelatin and 0.5% Triton-X-100, pH 7.4. Nuclei of cells were stained with TO-PRO-3 (Molecular Probes, 1:500). Coverslips were mounted in Mowiol+ (0.1M Tris-HCl pH8.5, 25% glycerol, 10% w/v mowiol 4-88 and 0.1% anti-fading w/v 1.4-diazabicyclo-(2.2.2)-octane). Images were acquired by confocal
laser scanning microscopy (Zeiss 410) with three different lasers emitting at 488, 543 and 633 nm to excite FITC, Cy3 and TO-PRO-3, respectively. For quantification experiments the cells were stained as described above except for the nuclear staining, which was performed with Hoechst (Biorad 10 μg/ml).

Quantification of cells

SK-N-SH cells were quantified by hand with ImagePro software (Media Cybernetics, Silver Springs, MD). Images were acquired with a Sony XC-77CC black/white camera through a Zeiss axioskop with a Plan-Neofluar objective (40x oil lens). For each coverslip, a Cy3 image and the corresponding Hoechst image were acquired in five fields. The experiment was performed in triplicate. The total number of cells was quantified by counting the nuclei, which were visualised by Hoechst staining. The number of transduced cells was quantified by counting the immunopositive cells in the Cy3 images. In an overlay of the Hoechst and Cy3 images, fragmented nuclei in immunopositive cells were counted. After summation of the number of cells of five fields per coverslip, averages were calculated for three coverslips per condition. A two-factor ANOVA, based on \( \alpha = 0.05 \) was performed for each graph. In case of significant effects in virus and time or in virus-time interaction, multiple comparison was performed to find significance between the different groups. The experiment was repeated in duplicate, which gave similar results.

RESULTS

UBB\(^{-1}\) has lost the ability to participate in tagging proteins for degradation by the proteasome

Recombinant His-tagged UBB\(^{-1}\) was produced in *Escherichia Coli*. The purified protein was used to study whether UBB\(^{-1}\) is able to conjugate proteins in a rabbit reticulocyte cell-free system (Hershko
UBB\(^*\) or wtUb were mixed with fraction II of rabbit reticulocyte lysate in the presence or absence of ATP. The reactions were stopped at different times (Fig. 1). WtUb was covalently conjugated to proteins only in the presence of ATP, as indicated by the appearance of Ub-containing bands with a molecular mass higher than monomeric Ub (8 kDa). When wtUb was substituted with UBB\(^*\) (11 kDa), no conjugates were observed, indicating that this mutant Ub was not able to conjugate to proteins. These results are consistent with the fact that UBB\(^*\) lacks a functional carboxyl-terminal residue (glycine 76) (Bamezai and Breslow, 1991).

**UBB\(^*\) is not degraded by the ATP-dependent proteasome pathway**

UBB\(^*\) is an abnormal protein: as such, it could be a substrate of the ubiquitin-proteasome dependent degradation pathway. We tested this possibility in the fraction II in vitro system. Radioiodinated UBB\(^*\) was incubated with fraction II and Ub in the presence and absence of ATP. As positive and negative controls for the degradation reaction, we used radioiodinated lysozyme substrates and wtUb, respectively (Fig. 2). Figure 2A shows that lysozyme was efficiently degraded in an ATP-dependent manner. WtUb (Fig. 2B) was not significantly degraded either with or without ATP. With radioiodinated UBB\(^*\) (Fig. 2C) we observed no ATP-dependent proteasomal degradation of the mutant protein. However, UBB\(^*\) was degraded in an ATP- and proteasome-independent reaction, possibly by a proteolytic activity present in fraction II. In these experiments, lysozyme is clearly the only protein that is degraded by an ATP-dependent proteasomal activity (compare the final amount of protein degraded in Fig. 2D). The results in Fig. 2 indicated that UBB\(^*\) was not a substrate of the ATP-dependent ubiquitin-proteasome pathway in the in vitro system.

**UBB\(^*\) is ubiquitinated in neuronal cells**

To determine whether UBB\(^*\) is processed in cells, we transduced the human neuroblastoma cell line SK-N-SH with adenoviral vectors (Hermens et al., 1997) encoding UBB\(^*\) (AdUBB\(^*\)) or wtUb (AdwtUb) as a control. Expression of both constructs was driven by the CMV promoter to ensure high expression of the proteins of interest. SK-N-SH cells were infected with AdUBB\(^*\) or AdwtUb with an MOI of 10 for 2 h, resulting in high expression of either protein with an
efficiency of more than 60% of the cells. Cultures were harvested 1 day after infection. Western blots of transfected cells probed with either anti-UBB+1 or anti-wtUb antibodies confirmed production of the proteins. In the Ad-wtUb infected cells, a monomeric Ub band with an approximate molecular weight of 8 kDa (Fig. 3) and a high molecular mass smear, representing multiple ubiquitinated proteins, were detected. AdUBB+1-infected cell lysates probed with UBB+1 antibody showed an 11 kDa UBB+1 band and higher molecular mass bands (+/- 19 kDa and 36-50 kDa) most likely representing ubiquitinated forms of UBB+1 (Fig. 3, asterisks). Ubiquitination of UBB+1 is likely to occur, since the protein contains a lysine at position 48, which is known to be a target for ubiquitination (Pickart, 1998). Lam et al. have recently shown that in 293T cells transfected with UBB+1, the mutant ubiquitin is also ubiquitinated (Lam et al., 2000). We found a more pronounced ubiquitination of UBB+1 than that reported by Lam et al. (Lam et al., 2000), which is most likely due to the efficient transduction by the Ad vector.

Fig. 2 UBB+1 is not degraded by the proteasomal machinery. Degradation was determined as the acid soluble material released during the course of the reaction and is depicted as percentage of protein after incubation with reconstituted reticulocyte lysate in the presence (△) and absence (■) of ATP (+/- SE). A Time course of the degradation of iodinated lysozyme. B Time course of the degradation of iodinated wtUb. C Time course of the degradation of iodinated UBB+1. D Three-hour incubation results from a direct comparison of the degradation rates. UBB+1 was not a substrate of the ubiquitin-proteasome pathway.
Fig. 3 UBB* is ubiquitinated. Western blot on transduced SK-N-SH cell lysates. Left lane: AdUBB*+1-infected SK-N-SH cells after 1 day of infection probed with anti-UBB* showing that UBB* (11 kDa, arrow) is ubiquitinated (* = ubiquitinated forms of UBB*). As a control, a blot on LacZ transduced cell lysate was probed with anti-UBB*, which did not show bands (data not shown). Right lane: Control of AdwtUb-infected cell lysate probed with anti-wtUb, showing multiple ubiquitinated proteins.

Ubiquitinated UBB*+1 is stable

Pulse-chase assays were performed to investigate the stability of UBB*+1 and its ubiquitinated forms, since the protein might be degraded after ubiquitination. SK-N-SH cells were transduced with AdUBB*+1, then labeled with [35S]-methionine/cysteine for 4 h. Cells were harvested at different times after labeling.

Immunoprecipitation of AdUBB*+1-infected cell lysates with anti-UBB*+1 antibody resulted in a pattern of bands (Fig. 4A, left lane) similar to that observed on the Western blots of infected cells. Immunoprecipitation of AdUBB*+1 transduced cell lysates with anti-wtUb antibody also strongly suggest that UBB*+1 is ubiquitinated. Several discrete high molecular weight bands of multimeric UBB*+1 were observed, but the monomeric form of UBB*+1 was not detected with the anti-wtUb antibody (Fig. 4A, right panel). One possible explanation is a conformational difference between monomeric forms of UBB*+1 and wtUb, which would prevent UBB*+1 from being recognized by the anti-wtUb antibody used in this experiment.

All UBB*+1 conjugate bands were stable for more than 3 h, indicating that UBB*+1 and ubiquitinated UBB*+1 were barely degraded (Fig. 4B).
The specificity of the assay was confirmed by immunoprecipitation with anti-UBB⁺⁺ of AdLacZ- and AdwtUb-infected cell lysates. No bands as observed in Fig. 4B were detected in those experiments (data not shown).

**Fig. 4** UBB⁺⁺ is stable. **A** Immunoprecipitation of AdUBB⁺⁺-infected SK-N-SH cells. Lane 1: immunoprecipitation was done with anti-UBB⁺⁺; lane 2: with anti-wtUb. The anti-wtUb antibody does not recognise the UBB⁺⁺ band but does stain the higher bands, confirming the ubiquitination of UBB⁺⁺. **B** Pulse chase assay on AdUBB⁺⁺-infected SK-N-SH cells. Cells were chased at different times after infection (t in hours). The image was acquired with a Phosphorimager after immunoprecipitation (IP) with anti-UBB⁺⁺ antibody or preimmune serum was performed. UBB⁺⁺ and its ubiquitinated forms were barely degraded after 3 hours.

**UBB⁺⁺ overexpression induces cell death**

It has been shown that UBB⁺⁺ inhibits the 26S proteasome in a cell-free system (Lam et al., 2000). Inhibition of the proteasome has been found to cause apoptosis in several cell lines (Pasquini et al., 2000; Qiu et al., 2000). To study the possible toxicity induced by UBB⁺⁺ in neuronal cells, SK-N-SH cells were transduced with either of the adenoviral vectors AdUBB⁺⁺, AdwtUb, or AdLacZ as a control. Cells were fixed at different times after transduction, stained for proteins with fluorescent antibodies, and analyzed by confocal laser scanning microscopy.
SK-N-SH cells transduced with UBB\(^{+1}\) and immunopositive for anti-UBB\(^{+1}\) staining were morphologically different from cells transduced with wtUb. UBB\(^{+1}\) positive cells exhibited vesicle-like extensions (Fig. 5A), whereas wtUb and LacZ transduced cells had a normal appearance (Fig. 5B). Cells were also stained with the nuclear dye TO-PRO, revealing occasional nuclear fragmentation in UBB\(^{+1}\) transduced cells (Fig. 6), but no fragmentation of nuclei was seen in LacZ or wtUb transduced cells (data not shown). Both vesicle-like extensions and nuclear fragmentation are reminiscent of apoptosis. From 1 to 6 days after transduction, fewer UBB\(^{+1}\) immunopositive cells were observed whereas the number of wtUb transduced cells remained stable over time, indicating that UBB\(^{+1}\) transduced cells had died (Fig. 7). Quantification of the cells after AdUBB\(^{+1}\) infection revealed a clear and significant decrease (p = 0.012) in the total number of cells on day 6 vs. day 1, whereas no significant decrease was observed in AdLacZ- or AdwtUb-infected cells (Fig. 8A). The number of immunopositive UBB\(^{+1}\) transduced cells significantly decreased (p=0.014) on day 6 after infection (Fig. 8B), indicating
that the decrease in the total number of cells was due to the death of UBB\textsuperscript{+1} transduced cells. The virus-time interaction of the analysis for the number of immunopositive cells was also significant \((F=2.63, p=0.04)\). Moreover, the immunopositive UBB\textsuperscript{+1} transduced cells still present in the culture on day 6 after infection showed a significant percentage of fragmented nuclei compared with day 1 \((p=0.001)\). This was a clear and significant increase compared with the wtUb and LacZ transduced cells on day 6 \((p=0.001)\) (Fig. 8C). Western blots of AdUBB\textsuperscript{+1}-infected cell lysates showed a decrease in UBB\textsuperscript{+1} and its ubiquitinylated forms at later times after infection, due to cell death, whereas wtUb was expressed equally well (data not shown). These data indicate that overexpression of UBB\textsuperscript{+1} triggers apoptosis in neuronal cells.

![Image](image-url)

**Fig. 7** UBB\textsuperscript{+1} transduced cells die at later times after infection. SK-N-SH cells infected with Ad-LacZ, AdwtUb or AdUBB\textsuperscript{+1} at different times after infection (days 1, 3, and 6). Ad-LacZ-infected cells were stained with anti-Gal3, AdwtUb-infected cells with anti-wtUb, and AdUBB\textsuperscript{+1}-infected cells with anti-UBB\textsuperscript{+1} antibody. The number of UBB\textsuperscript{+1} transduced cells clearly decreases at later times after infection whereas the control infections show remarkably less cell death.
Fig. 8 Quantification of UBB<sup>+</sup> induced cell death

A Quantification of total number of cells by counting nuclei stained with Hoechst. The total amount of cells is significantly decreased 6 days after AdUBB<sup>+</sup> infection compared with day 1 (p = 0.012), whereas no significant decrease was observed in AdwtUb- or AdLacZ-infected cells.

B Quantification of Ad-virus transduced cells by counting immunopositive cells (stained for either LacZ, wtUb or UBB<sup>+</sup>). Two-factor ANOVA showed a significant virus-time interaction (F = 2.62, p = 0.04, p(time) = 0.06, p(virus) < 0.01). The amount of UBB<sup>+</sup> transduced cells significantly decreased 6 days after infection vs. day 1 (p = 0.014), indicating that UBB<sup>+</sup> transduced cells die.

C Quantification of fragmented nuclei of immunopositive transduced cells depicted as percentage of nuclei in immunopositive cells. UBB<sup>+</sup> transduced cells showed a significant percentage of fragmented nuclei (p = 0.001) on day 6 after infection, indicating that UBB<sup>+</sup> transduced cells had died in an apoptotic fashion.
DISCUSSION

Our results reveal that UBB\(^{+1}\) is defective in ubiquitinating proteins (Fig. 1). This mutant ubiquitin was not conjugated to proteins in reticulocyte fraction II, which contains all required elements of the ubiquitin-proteasome machinery. That UBB\(^{+1}\) is unable to conjugate to proteins is not surprising, since the carboxyl-terminal glycine at position 76 (Gly76) of Ub, which is lacking in UBB\(^{+1}\), is essential in conjugating to other proteins (Varshavsky, 1997). The lack of conjugating properties of UBB\(^{+1}\) indicates that the mutant protein is not able to participate in targeting proteins for degradation. This finding agrees with data on the inability of the Des-75-76 Ub molecule (lacking the Gly75 and 76 residues) to conjugate to substrates (Bamezai and Breslow, 1991) and the inability of UBB\(^{+1}\) to bind to E1-activating enzyme (Lam et al., 2000).

Furthermore, we show that UBB\(^{+1}\) is not degraded by the ATP-dependent proteasome in reticulocyte lysate (Fig. 2), nor in neuronal cells (Fig. 4B). Surprisingly, we found that although UBB\(^{+1}\) is ubiquitinated, it is refractory to degradation in neuroblastoma cells, as UBB\(^{+1}\) and its ubiquitinated forms were stable for at least 3 h (Fig. 4B). Normally, most proteins targeted for degradation by the proteasome by ubiquitination are degraded in minutes (Johnson et al., 1992; Lenk and Sommer, 2000).

If UBB\(^{+1}\) is not degraded by the proteasome, why is it ubiquitinated? One possibility is that UBB\(^{+1}\) is targeted for degradation by ubiquitination but somehow inhibits the proteasome (discussed later). On the other hand, ubiquitination of UBB\(^{+1}\) may not represent a targeting signal but be part of the preassembly of multi-Ub chains. In vivo, unanchored multi-Ub chains are formed by conjugating (E2) and ligating (E3) enzymes that are specific for Ub. This preassembly of multi-Ub chains enables fast multi-ubiquitination and subsequent degradation of target proteins (Mastrandrea et al., 1999). UBB\(^{+1}\) could theoretically be incorporated into a preassembled multi-Ub chain, as this is formed by conjugation to either Lys29 or Lys48 (Mastrandrea et al., 1999), which are both present in UBB\(^{+1}\). However, UBB\(^{+1}\) primed Ub chains will not be degraded by isopeptidase T (Wilkinson et al., 1995) as are normal multi-Ub chains because this enzyme requires a carboxyl-terminal Gly residue at the proximal end of the chain (Lam et al., 2000; Wilkinson et al., 1995).
Similarly, isopeptidase T is not effective in the disassembly of Des-75-76 Ub terminated chains (Amerik et al., 1997).

Our experiments show that high expression of UBB+1 induces massive cell death in human neuroblastoma cells, as seen by the sharp and significant decrease in the number of UBB+1-transduced cells in time (Fig. 8B). The decrease in UBB+1 immunopositive cells was accompanied by a decrease in total cell number (Fig. 8A), indicating that the UBB+1 transduced cells die. In addition, the morphology of UBB+1 immunopositive cells, i.e. vesicular extensions and nuclear fragmentation (Fig. 8C), show that UBB+1 transduced cells are dying in an apoptotic fashion, since apoptosis is normally characterised by blebbing, cell shrinkage, and nuclear condensation and fragmentation (McGahon et al., 1995).

The induction of cell death by UBB+1 is in line with the hypothesis that this mutant Ub inhibits the proteasome. Inhibition of the proteasome through other mechanisms has been shown to cause apoptosis in many studies, including apoptosis in neuronal cells. In cultured cerebellar granule cells, for example, inhibition of the proteasome by lactacystin results in apoptosis and activation of caspase-3 (Pasquini et al., 2000). In primary cultured cortical neurons, proteasome inhibitors carbobenzoxy-Leu-Leu-Leu-aldehyde and lactacystin both induced caspase-3 mediated apoptotic neuronal death (Qiu et al., 2000). Based on these results, the apoptotic death induced by UBB+1 can be explained by a direct inhibitory effect of UBB+1 on the proteasome.

In the pathology of AD, increasing evidence emerges for a role of apoptosis. However, the evidence is limited because cell loss is a dynamic process that almost certainly occurs over many years; therefore, neuropathologic studies may not have adequate sensitivity to detect relatively rapid processes such as apoptosis. In several studies, however, DNA laddering and terminal dUTP-mediated nick-end labeling were found in brains of AD patients vs. brains of nondemented control patients (Anderson et al., 2000; Su et al., 1994). Moreover, evidence for apoptosis decision cascades (Cotman, 1998) and a correlation between caspase activation and neurofibrillary tangle formation (Rohn et al., 2001) were recently found to be involved in AD.

It was reported recently that the proteasomal activity in brains of AD patients is diminished (Keller et al., 2000a). The activity of
the proteasome was significantly decreased in the parahippocampal gyrus, the superior and middle temporal gyri, and the inferior parietal lobe of AD brains, which are regions showing severe degenerative alterations in AD and UBB^{+1} staining. Moreover, ubiquitination of cerebral proteins was found to be defective in AD (Lopez Salon et al., 2000), suggesting that the Ub-proteasome degradation machinery is involved in the pathogenesis of AD. These data are, however, purely descriptive and do not indicate a molecular cause for the down-regulation of the proteasomal activity. In contrast, our earlier data on the neuronal accumulation of UBB^{+1} in AD patients (Van Leeuwen et al., 1998b), in combination with the data of Lam et al. (Lam et al., 2000) on the inhibition of the proteasome by UBB^{+1} and our present data on the stability of UBB^{+1} and induction of apoptotic cell death, strongly suggest that UBB^{+1} is a key protein in causing the proteasomal inhibition in AD brains.

In summary, we have shown that UBB^{+1} does not exhibit the degrading and conjugating properties of wtUb and that accumulating levels of this mutant protein in neuronal cells are detrimental and cause neuronal apoptosis. We also show that UBB^{+1} is ubiquitinated and barely degraded in neuronal cells. The finding that UBB^{+1} 1) is expressed in AD brains, 2) lacks the ability to ubiquitinate, 3) inhibits proteasomal activity (Lam et al., 2000) and 4) induces apoptotic cell death in neurons shows that this mutant Ub can interfere with normal neuronal functioning and probably contributes to neurodegeneration. Therefore, we propose that molecular misreading of the ubiquitin B gene is an early event in the pathogenesis of AD. The gradual and slow accumulation of UBB^{+1} protein will eventually inhibit the proteasomal activity in neurons, interfering with normal neuronal functions and resulting in neuronal loss.
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