

Inhibition of the ubiquitin-proteasome system in Alzheimer's disease

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Alzheimer's disease is the most common cause of dementia in the elderly. Although several genetic defects have been identified in patients with a family history of this disease, the majority of cases involve individuals with no known genetic predisposition. A mutant form of ubiquitin, termed Ub⁺¹, has been selectively observed in the brains of Alzheimer's patients, including those with non-familial Alzheimer's disease, but it has been unclear why Ub⁺¹ expression should be deleterious. Here we show that Ub⁺¹ is an efficient substrate for polyubiquitination *in vitro* and in transfected human cells. The resulting polyubiquitin chains are refractory to disassembly by deubiquitinating enzymes and potentially inhibit the degradation of a polyubiquitinated substrate by purified 26S proteasomes. Thus, expression of Ub⁺¹ in aging brain could result in dominant inhibition of the Ub-proteasome system, leading to neuropathologic consequences.

The conserved protein ubiquitin (Ub) functions as a covalent proteolytic signal. Conjugation of intracellular proteins to a polyUb chain is a signal for targeting to 26S proteasomes, which degrade these substrates while sparing Ub (1, 2). The first Ub is linked to the substrate through an isopeptide bond that joins the C terminus of Ub (G76) to an internal lysine residue of the substrate. To generate an efficient proteasomal targeting signal, additional Ubs are linked to the first one by means of K48-G76 isopeptide bonds between successive Ub molecules (3–5). The Ub-proteasome system regulates the levels of a host of vital regulatory proteins in eukaryotic cells, including cyclins, cyclin-dependent kinase inhibitors, transcription factors, and oncoproteins (1, 2). In accordance with a requirement for the proper homeostasis of such substrates, a functional Ub-proteasome system is essential for the viability of eukaryotic cells (1, 2, 6).

The molecular pathological hallmarks of Alzheimer's disease (AD) are intracellular neurofibrillary tangles and extracellular amyloid (A β) plaques (7). The A β in plaques arises from specific processing of amyloid precursor protein that is positively regulated by the presenilins PS1 and PS2 (8, 9). PS1 and PS2 recently have been shown to be substrates of the Ub-proteasome system (10–13). Although certain mutations in the genes encoding amyloid precursor protein and PS1/PS2 are known to confer a genetic predisposition to AD, such mutations are absent in the majority of patients with AD (7). The Ub-proteasome system was first implicated in AD through the immunohistochemical observation of Ub-conjugated tau protein in neurofibrillary tangles (14, 15). However, although the presence of Ub in abnormal aggregates is characteristic of AD and several other neurodegenerative diseases (14–16), the origin of these structures and their mechanistic relationship to disease onset and progression remain unclear.

Recently, van Leeuwen and coworkers (17, 18) discovered that a mutant form of Ub, termed Ub⁺¹, is selectively expressed in the brains of AD patients. Expression of Ub⁺¹ occurs through a phenomenon known as molecular misreading, in which non-DNA-encoded dinucleotide deletion(s) are found in mRNA within or adjacent to GAGAG motifs (17–19). Van Leeuwen *et*

al. (17) localized a dinucleotide deletion to the first coding unit of a polyUb transcript, causing replacement of the C-terminal residue of Ub (G76) with a 20-residue (+1) extension. Those investigators used several approaches to show that Ub⁺¹ was present in the brains of AD patients, including those without known predisposing factors (17). In contrast, Ub⁺¹ was present at a significantly reduced level in the brains of nondemented control patients (17, 18). Although these results demonstrated a striking correlation between Ub⁺¹ expression and AD pathogenesis, it was unclear why Ub⁺¹ expression should be deleterious. Here we demonstrate a mechanism by which Ub⁺¹ expression could lead to dominant inhibition of the Ub-proteasome system.

Materials and Methods

Proteins and Enzymes. Ub⁺¹ was chemically synthesized by using ≈ 0.5 g fluorenylmethoxycarbonyl-Gln-Wang resin functionalized at 0.2 mmol/g (20). The overall yield after purification was 5–10%. The Ub-ribosomal fusion protein Ub-CEP52 was prepared similarly (20). Ub5⁺¹ was synthesized by using purified recombinant ubiquitin conjugating enzyme-25K to conjugate preassembled, K48-linked Ub₄ (18 μ M) to K48 of Ub⁺¹ (26 μ M). The conditions of this reaction and the synthesis of Ub₄ have been described (5). Ub5⁺¹ was purified by cation exchange chromatography (5). Purified recombinant glutathione S-transferase (GST)-Ub carboxyl-terminal hydrolase-D (UCH-D) was a gift of M. Bownes (University of Edinburgh). Isopeptidase T (21) and ubiquitin activating enzyme (E1) (5) were purified to homogeneity from bovine erythrocytes.

Extract Preparation. Human embryonic kidney 293T cells were lysed in a buffer containing 20 mM Tris (pH 7.6), 0.1% NP-40, 0.5 mM EDTA, 1 mM PMSF, 5 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 50 μ M tosyl lysyl chloromethyl ketone, and either 0.5 mM DTT (for assays of deubiquitination) or 1 mM N-ethyl maleimide (to detect polyUb chains). Lysates were centrifuged at 13,000 \times g for 10 min; the supernatant (soluble extract) was assayed.

Deubiquitination Assays. Incubations with GST-UCH-D contained 20 nM enzyme and 4 μ M of either Ub⁺¹ or Ub-CEP52.

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Abbreviations: AD, Alzheimer's disease; E1, ubiquitin activating enzyme; GST, glutathione S-transferase; Ub, ubiquitin; UCH-D, Ub carboxyl-terminal hydrolase-D.

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In some cases the enzyme was preinactivated with *N*-ethyl maleimide. Incubations (pH 7.6, 37°C) were quenched after 2 h; reactants/products were detected by blotting with Ub antibodies. Assays of purified isopeptidase T (pH 7.6 and 37°C) contained 20 μ M of either Ub⁵⁺¹ or Ub₄ and 0.1 to 1 μ M enzyme. Incubations were quenched after 30 min and reactants/products were detected by Western blotting with +1 or Ub antibodies. For assays of deubiquitination in 293T cell extracts, soluble lysate was prepared by using DTT (above). The lysate was diluted to a protein concentration of 1 mg/ml and incubated at 37°C with 1 μ M of Ub₄ or Ub⁵⁺¹. In the experiment shown in Fig. 3D, Ub₄ and Ub⁵⁺¹ were pretreated with a purified deubiquitinating enzyme, YUH-1. In the case of Ub₄, this treatment removes an extra residue (D77) in the proximal Ub that resulted from the synthetic procedure (5). In the case of Ub⁵⁺¹, YUH-1 (equivalent to UCH-D) has no effect (see *Results*). Aliquots were removed at increasing times up to 3 h and analyzed by blotting with Ub antibodies.

Proteasome Assays. 26S proteasomes (2.5 nM) purified from bovine erythrocytes (22) were incubated with 20 nM of the synthetic substrate ³⁵S-Ub₅-dihydrofolate reductase in the presence of MgATP, with or without increasing concentrations of Ub₄ or Ub⁵⁺¹ (5). Degradation was monitored as the appearance of acid-soluble radioactivity at a time (20 min) determined to be in the linear range of the assay. *K_i* values were calculated by assuming competitive inhibition, using *K_m* = 17 nM (*K_m* value determined for the proteasome preparation used in these experiments).

E1 Assay. E1 was assayed based on its ability to form a thiol ester adduct with ¹²⁵I-Ub, which was detected by autoradiography after resolution by SDS/PAGE under nonreducing conditions. Purified E1 was incubated with 2 μ M ¹²⁵I-Ub in the presence of MgATP. To monitor the binding of Ub⁺¹, increasing concentrations of the unlabeled synthetic protein were mixed with ¹²⁵I-Ub before initiating adduct formation by adding 0.1 μ M E1 (23). For the control, increasing concentrations of unlabeled wild-type Ub were used. The level of the labeled adduct was quantitated by PhosphorImage analysis.

Ub⁺¹ and Ub Plasmids. Synthetic genes encoding Ub⁺¹, Ub⁺¹ with a C-terminal H₆ tag, and Ub, were generated by PCR and cloned into the *Cla*I/*Eco*RV sites of the tetracycline-off pTet-Splice vector from Life Technologies, Grand Island, NY (24). In Ub⁺¹-H₆ the final amino acid was replaced with the sequence AAALHHHHHH. As part of the cloning procedure, residue 2 was mutated from Gln to Ala in all three coding sequences.

Transfection. Subconfluent 293T cells were cotransfected with the indicated pTet-Splice vector together with pTet-tTak encoding a tet-repressible hybrid activator (4 μ g each plasmid/well on 6-well plates, Lipofectamine method). Transfection efficiency was 10–20%. Cells were lysed in buffer containing *N*-ethyl maleimide (above) after 26 h of transfection using 40 μ l of buffer per well of a 6-well plate.

Nickel Binding. Soluble lysate from transfected 293T cells (above) containing between 200 and 675 μ g of protein (see the figure legends) was applied to 10 μ l of charged His-bind resin (Novagen). The resin was washed with lysis buffer, and then eluted with SDS/PAGE sample buffer. Aliquots of the starting material, unbound fraction, and bound fraction were analyzed by blotting with anti-Ub antibodies.

Antibodies and Western Blotting. Polyclonal Ub antibodies (25) were affinity-purified. Polyclonal antibodies specific for Ub⁺¹ were raised in rabbits against synthetic +1 peptide; the IgG

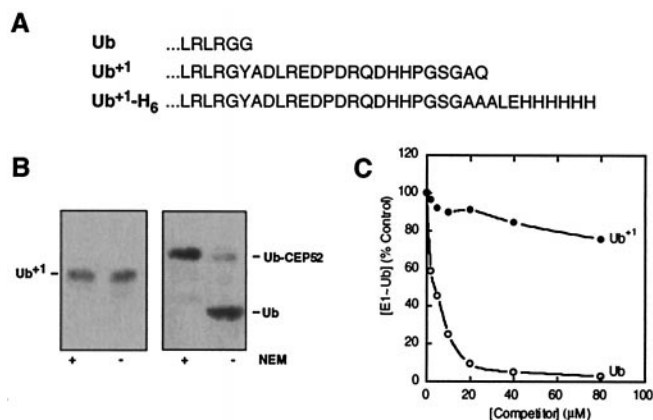


Fig. 1. Ub⁺¹ is refractory to processing and activation. (A) Unique sequence of Ub⁺¹. The sequences of the extreme C termini of Ub, Ub⁺¹ (17), and Ub⁺¹-H₆ (see *Materials and Methods*) are shown. (B) Processing (Ub blots). (Left) Ub⁺¹ was incubated (see *Materials and Methods*) with GST-UCH-D that had been preinactivated (or not) with *N*-ethyl maleimide (NEM) (as indicated). (Right) Control: the same enzyme processed Ub-CEP52, a chemically synthesized Ub-ribosomal fusion protein. (C) Activation. Increasing concentrations of Ub (○) or Ub⁺¹ (●) were mixed with 2 μ M ¹²⁵I-Ub before initiating assays of E1-Ub thiol ester formation with purified E1 (see *Materials and Methods*).

fraction was used in blotting experiments. Immunocomplexes were detected by using enhanced chemiluminescence.

Results

Ub⁺¹ Does Not Inhibit Ub Conjugation. Because Ub is synthesized as a fusion either with itself or with a ribosomal protein, Ub activation and conjugation strictly depend on the exposure of G76 through processing by deubiquitinating enzymes (4, 26). In the case of Ub⁺¹, G76 is replaced with the 20-residue +1 peptide (Fig. 1A; ref. 17). To test the potential of Ub⁺¹ to enter the conjugation phase of the Ub-proteasome system, we first addressed whether Ub⁺¹ was processed by a purified deubiquitinating enzyme, UCH-D (20). We found that the +1 peptide of Ub⁺¹ was not removed by a GST-fused form of UCH-D (Fig. 1B Left), whereas the same enzyme efficiently processed a Ub-ribosomal fusion protein (Fig. 1B Right). Ub⁺¹ also failed to undergo processing in a crude extract prepared from human embryonic kidney 293T cells (data not shown) despite the presence of a high level of deubiquitinating activity in this extract (see below).

To test whether Ub⁺¹ could interfere with Ub conjugation, we determined whether Ub⁺¹ could bind to E1. E1 catalyzes the obligatory first step in all ubiquitination reactions and forms a catalytic thiol ester intermediate with Ub (1, 2). Any Ub⁺¹ binding to E1 can be monitored through inhibition of E1-Ub thiol ester formation (23). As shown in Fig. 1C (●), Ub⁺¹ exhibited negligible binding in comparison to wild-type Ub (○). The lack of Ub⁺¹ processing by cellular deubiquitinating enzymes (Fig. 1B) and the failure of Ub⁺¹ to bind E1 (Fig. 1C) are in agreement with earlier demonstrations that G76 is a key specificity element in Ub processing and activation (4, 26–30). It is therefore most unlikely that Ub⁺¹ will inhibit the conjugation of wild-type Ub to cellular substrates.

Assembly of Ub⁺¹-Capped PolyUb Chains in Cultured Mammalian Cells. Although Ub⁺¹ did not inhibit the conjugation phase of the proteolytic system, we reasoned that inhibition of the Ub-proteasome system could still occur through the accumulation of aberrant polyUb chains containing Ub⁺¹. In general, a substrate must be conjugated to a K48-linked polyUb chain to be efficiently targeted to proteasomes (1–5). In the pathway for

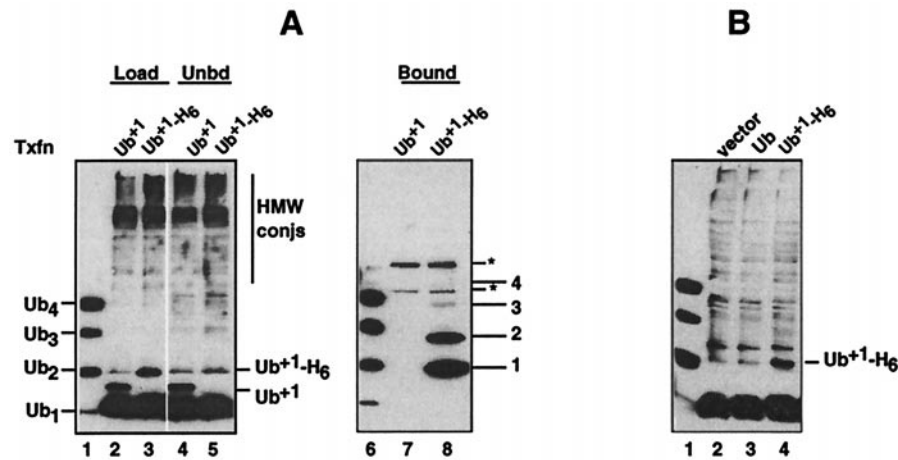


Fig. 2. Assembly of Ub⁺¹-capped chains in mammalian cells (Ub blots). (A) Experiment. Human embryonic kidney 293T cells were transfected with plasmids specifying unmodified Ub⁺¹ (lanes 2, 4, and 7) or Ub⁺¹-H₆ (lanes 3, 5, and 8). (Left) 200 μg of soluble extract protein was incubated with 10 μl of nickel beads; 10% of the starting lysate (lanes 2 and 3) and unbound fraction (lanes 4 and 5) were analyzed. The entire bound fraction deriving from 675 μg of lysate protein was analyzed in lanes 7 and 8. * denote cross-reacting bands that bound to the beads. Lanes 1 and 6, mixture of authentic polyUb chains. Ub⁺¹-H₆ (band 1) and presumptive Ub⁺¹-H₆-capped chains (bands 2–4) are indicated on the right. Conjs-conjugates. (B) Controls. Human 293T cells were transfected with empty vector (lane 2), vector specifying wild-type Ub (lane 3), and vector specifying Ub⁺¹-H₆ (lane 4). Soluble extracts were analyzed as in lanes 2 and 3 of A; lane 1, chain standard. The band in lanes 2 and 3, which comigrates with Ub⁺¹-H₆ and which does not bind to nickel beads (lanes 3 versus 5, A), is probably wild-type Ub₂.

recycling of Ub, the chain is first released from the substrate; the resulting unanchored chain ultimately is disassembled by isopeptidase T (Ubp14 in yeast), a deubiquitinating enzyme whose activity requires an unblocked G76 residue at the proximal chain terminus (21, 30). (The proximal terminus refers to that end of the chain that could normally be conjugated to a substrate protein.) Inhibition of the disassembly of unanchored polyUb chains is deleterious, principally because the chains compete with polyubiquitinated substrates for binding to proteasomes (5, 30).

Besides arising as intermediates in the degradation of polyubiquitinated substrates, unanchored chains can be assembled *de novo* (4, 30, 31). Because Ub⁺¹ retains K48 and other lysine residues implicated in polyUb chain assembly (4), it could act as a substrate for conjugation of wild-type Ub; the resulting Ub⁺¹-capped polyUb chains may escape disassembly by isopeptidase T because of the absence of G76 in Ub⁺¹. Similarly, when yeast cells are engineered to overexpress a C-terminally truncated version of Ub that lacks the C-terminal GG dipeptide, stable polyubiquitinated forms of this truncated protein accumulate and inhibit proteasomes, leading to a growth defect (30). Expression of Ub⁺¹ thus could lead to the accumulation of stable polyUb chains, causing inhibition of proteasomes.

To demonstrate intracellular assembly of Ub⁺¹-capped chains, we transfected 293T cells with a gene encoding Ub⁺¹, with or without a C-terminal hexahistidine tag (Fig. 1A). Western blot analysis of transfected cell lysates (with Ub antibodies) showed that each protein was expressed (Fig. 2A, lanes 2 and 3); the bands designated Ub⁺¹ and Ub⁺¹-H₆ in Fig. 2 were absent in lysates of cells transfected with empty vector or a control plasmid specifying wild-type Ub (Fig. 2B). In cells transfected with the Ub⁺¹-H₆ gene, analysis of proteins retained on nickel beads showed the presence of higher forms of Ub⁺¹ with the mobilities expected for Ub₂⁺¹ and Ub₃⁺¹, with a faint band at the position of Ub₄⁺¹ (Fig. 2A, lane 8). Specificity was demonstrated by the absence of these species in the bound fraction from cells that received the untagged Ub⁺¹ gene (Fig. 2A, lane 7). We conclude that Ub⁺¹ is indeed assembled into polyUb chains within cells, perhaps by a chain assembly pathway that efficiently uses free, monomeric Ub as a substrate (31). The abundance of Ub⁺¹-capped chains, although low, is striking in view of the weak

expression of Ub⁺¹ (Fig. 2A, lanes 2 and 3) and the short duration of the experiment (26 h). Our results therefore suggest that Ub⁺¹-capped chains could accumulate to significant levels in aging brain even though molecular misreading is an inefficient process (17–19).

Ub⁺¹-Capped Chain Strongly Resists Disassembly. As discussed above, the facile accumulation of Ub⁺¹-capped chains in 293T cells (Fig. 2) may reflect resistance to disassembly by cellular deubiquitinating enzymes because of replacement of G76 with the +1 peptide. To test this hypothesis directly, we used a purified Ub conjugating enzyme to link preassembled Ub₄ to K48 in Ub⁺¹ (see *Materials and Methods*). The purified product, termed Ub₅⁺¹, carried one Ub⁺¹ moiety at its proximal terminus (Fig. 3A). We first determined whether Ub₅⁺¹ could be disassembled by isopeptidase T, which is the principal enzyme responsible for the disassembly of unanchored polyUb chains (21, 30). As shown in Fig. 3B, Ub₅⁺¹ was not disassembled by purified isopeptidase T at any concentration of this enzyme tested. In contrast, isopeptidase T readily processed the same concentration of Ub₄ (Fig. 3C). Ub₅⁺¹ also was disassembled ≈100-fold more slowly than Ub₄ in 293T cell extract (Fig. 3D), indicating that other deubiquitinating enzymes (26) are also deficient in dismantling Ub⁺¹-capped chains. The extreme stability of Ub₅⁺¹ (Fig. 3) suggests a high likelihood that Ub⁺¹-capped chains will accumulate in cells, even though they are likely to be generated at a slow rate (above).

Ub⁺¹-Capped PolyUb Chain Potently Inhibits 26S Proteasomes. We next tested the ability of Ub₅⁺¹ to inhibit the degradation of a polyubiquitinated model substrate (5) by purified 26S proteasomes. As shown in Fig. 4 (●) Ub₅⁺¹ was a potent inhibitor, with a K_i of 350 nM. The potency of Ub₅⁺¹ was approximately 3-fold greater than that of Ub₄ (Fig. 4, ● versus ○). The proteasomal signaling potential of K48-linked polyUb chains is length-dependent; Ub₅ is expected to bind about 2-fold more tightly than Ub₄ (5). The slightly augmented affinity of Ub₅⁺¹, in comparison to a normal chain of the same length, may reflect a small effect caused by the absence of a carboxylate at residue 76 (5).

Discussion

Our finding that Ub⁺¹ is polyubiquitinated to form stable polyUb chains provides a mechanistic paradigm for interpret-

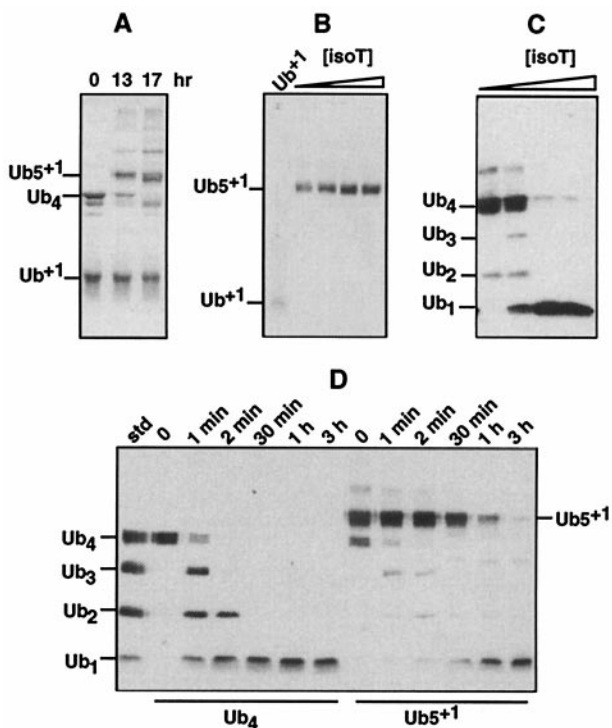


Fig. 3. Ub⁺¹-capped chains resist disassembly. (A) Synthesis of Ub⁵⁺¹ (Coomassie-stained gel). The conjugation reaction (see *Materials and Methods*) was sampled at the indicated times. (B) Disassembly of Ub⁵⁺¹ by isopeptidase T (+1 blot). First lane: Ub⁺¹ standard. Next four lanes: Ub⁵⁺¹ was incubated with increasing concentrations of purified isopeptidase T (left to right: none, 0.1 μM, 0.5 μM, 1 μM) as described in *Materials and Methods*. (C) Disassembly of Ub₄ by isopeptidase T (Ub blot). Conditions were identical to last four lanes of B, except the chain was Ub₄. (D) Disassembly of Ub₄ versus Ub⁵⁺¹ in mammalian cell lysate (Ub blots). Soluble lysate from 293T cells, prepared using DTT, was incubated with Ub₄ or Ub⁵⁺¹ for the indicated times (see *Materials and Methods*). First lane, polyUb chain standard. A trace of Ub₄, present as a contaminant in Ub⁵⁺¹, was rapidly disassembled in the Ub⁵⁺¹ incubation.

ing prior findings (14–17). Our results indicate that unmodified Ub⁺¹ is unlikely to be detrimental (Fig. 1); in contrast, polyubiquitinated forms of Ub⁺¹ potentially inhibit proteasomes (Fig. 4), providing a likely mechanism of toxicity. Age-dependent accumulation of Ub⁺¹-capped chains is predicted based on facile polyubiquitination of Ub⁺¹ (Fig. 2) and strong resistance of the resulting chains to disassembly (Fig. 3). Taken together, our results demonstrate a mechanism whereby an AD/age-related change in the properties of the Ub-proteasome system could lead to neuropathological consequences. Because inhibition by the mechanism described here relies on the modification of the mutant protein by wild-type Ub, Ub⁺¹ should act in a dominant negative fashion. This feature is critical in view of the low efficiency of the misreading process (17–19). Van Leeuwen and coworkers (17) estimated that frame-shifted UbB transcripts comprise between 1% and 10% of the total UbB transcripts in the brains of AD patients. Our finding that Ub⁺¹-capped polyUb chains are 100-fold more resistant to disassembly than normal chains suggests that the aberrant chains could accumulate to significant levels in the brain despite the low efficiency of molecular misreading.

We have not yet detected Ub⁺¹-capped chains in brain extracts from AD patients, a result that we attribute to slow postmortem disassembly of the aberrant chains (compare Fig. 3D). Consistent with this explanation, we found that normal polyUb chains and Ub-conjugated proteins are virtually absent from such

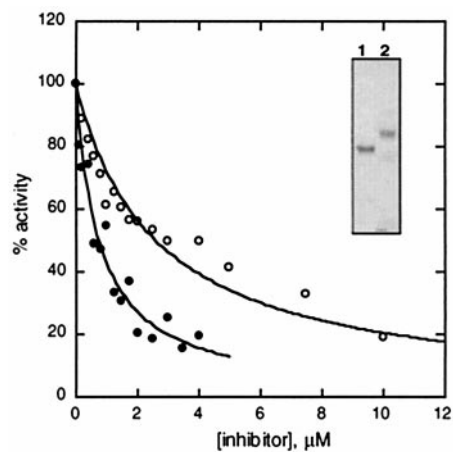


Fig. 4. Ub⁵⁺¹ potently inhibits 26S proteasomes. Purified 26S proteasomes were incubated with ³⁵S-Ub₅ dihydrofolate reductase, with or without the indicated chain inhibitor (○, Ub₄; ●, Ub⁵⁺¹) as described in *Materials and Methods*. Initial rates of degradation are expressed relative to a control without inhibitor. The lines are fits assuming competitive inhibition (see *Materials and Methods*), with $K_i = 350 \pm 30$ nM (Ub⁵⁺¹) or 1.2 ± 0.08 μM (Ub₄). (Inset) Chain inhibitors (Coomassie-stained gel): 1 μg Ub₄ (lane 1); 0.75 μg Ub⁵⁺¹ (lane 2).

extracts (data not shown). The detection of polyubiquitinated Ub⁺¹ in neurons is thus likely to require the development of a specific cell culture or animal model. The availability of such a model also should allow demonstration of the intracellular proteasome inhibition that should occur upon accumulation of capped chains that are four or more Ubs long (5, 30).

The presenilins, which positively regulate the γ-secretase (Aβ generating) cleavage of amyloid precursor protein (8, 9), are substrates of the Ub-proteasome system (10–13). It is therefore possible that a progressive, age-dependent inhibition of 26S proteasomes by Ub⁺¹-capped chains could contribute to AD pathogenesis through effects on presenilin levels. Additionally, proteasome inhibitors cause the formation of Ub-containing “aggresomes” in cultured cells (13)—inclusions that may be functionally related to the filamentous Ub-containing inclusions seen in the brains of patients with neurodegenerative diseases (14–17). Finally, inhibition of proteasomes would inhibit the degradation of misfolded proteins of the endoplasmic reticulum, leading to an increased demand on resident chaperones of this compartment (reviewed in ref. 32) and the possibility of increased amyloid precursor protein processing through competitive effects at the level of BiP/Grp78 (33). Inhibition of 26S proteasomes thus might contribute to both Aβ and tau pathology. The presence of Ub⁺¹ in tau-related neurofibrillary tangles in progressive supranuclear palsy (34) suggests that a similar mechanism could contribute to pathogenesis in other neurodegenerative diseases. We note that gracile axonal dystrophy is caused by an intragenic deletion in the gene encoding a deubiquitinating enzyme (35). Inactivation of this enzyme could lead to elevated levels of linear polyUb, which also inhibits proteasomal proteolysis (5). Inhibition of proteasomes thus could contribute to neuropathogenesis through pathways that are related to, but distinct from, the mechanism documented here.

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