Structural Basis of E2–25K/UBB⁺¹ Interaction Leading to Proteasome Inhibition and Neurotoxicity

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E2–25K/Hip2 is an unusual ubiquitin-conjugating enzyme that interacts with the frameshift mutant of ubiquitin B (UBB⁺¹) and has been identified as a crucial factor regulating amyloid-β neurotoxicity. To study the structural basis of the neurotoxicity mediated by the E2–25K–UBB⁺¹ interaction, we determined the three-dimensional structures of UBB⁺¹, E2–25K and the E2–25K/ubiquitin, and E2–25K/UBB⁺¹ complex. The structures revealed that ubiquitin or UBB⁺¹ is bound to E2–25K via the enzyme MGF motif and residues in α9 of the enzyme. Polyubiquitylation assays together with analyses of various E2–25K mutants showed that disrupting UBB⁺¹ binding markedly diminishes synthesis of neurotoxic UBB⁺¹-anchored polyubiquitin. These results suggest that the interaction between E2–25K and UBB⁺¹ is critical for the synthesis and accumulation of UBB⁺¹-anchored polyubiquitin, which results in proteasomal inhibition and neuronal cell death.

The pathological hallmarks of neurodegenerative diseases are the accumulation of abnormal proteins, neuritic plaques, neuropil threads, and neurofibrillary tangles (1). Although all aspects of this aggregation are considered harmful, the aberrant protein accumulation is likely the principal cause of disease. Among these proteins are presenilin-processed amyloid-β peptide (Aβ), a mutant form of ubiquitin (UBB⁺¹), and polyglutamine-expanded huntingtin (2). UBB⁺¹ was first identified as a frameshift mutant of the ubiquitin (Ub) B protein in the brains of neurodegenerative disease patients (3) and is composed of a Ub moiety (75 residues) with a 19-residue C-terminal extension. Neither Aβ nor UBB⁺¹ is found in young patients not suffering from dementia, but they are observed in older Alzheimer disease patients (4). The genes from which UBB⁺¹ mRNAs are transcribed contain several GAGAG motifs, and dinucleotide deletions (AGA) from within the GAGAG motif result in an abnormal C-terminal sequence. Normally these aberrant proteins are removed by the Ub-proteasome system (UPS), which executes the proteolytic degradation of aberrant proteins via a Ub-tagging mechanism (3, 5).

Within the UPS, Ub tagging of target molecules entails enzymatic reactions catalyzed by the E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes. Once E3 tags a target molecule with mono- or polyUb, the tagged molecule is recognized by the 26 S proteasome and degraded (6). In the healthy brain both β-amyloid precursor protein and UBB⁺¹ molecules are targets for the UPS and are degraded by the 26 S proteasome (7, 8). In the brains of Alzheimer patients, however, both UBB⁺¹ and Ub are present within aggregation plaques also containing β-amyloid precursor protein, which is indicative of UPS dysfunction (9, 10). When at normal basal levels, UBB⁺¹ can be removed by the UPS. But when its expression is up-regulated, UBB⁺¹ inhibits the 26 S proteasome in a dose-dependent manner, resulting in the accumulation of aberrant proteins (11). The aberrant C terminus of UBB⁺¹ prevents its activation and, therefore, subsequent ligation to substrates due to

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The atomic coordinates and structure factors (codes 2KX0, 3K9P, and 3K9O) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The assigned NMR resonances of UBB⁺¹ have been deposited on the Biological Magnetic Resonance Bank (BMRB) under BMRB code 16895.

The nucleotide sequence reported in this paper has been submitted to the Swiss-Prot protein database (29) and to GenBank (accession nos. P61086 and P62988). These authors contributed equally to this work.

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4 The abbreviations used are: Aβ, amyloid-β peptide; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; GFPu, green fluorescent protein fused to a short degron; NOESY, two-dimensional nuclear Overhauser effect (NOE) spectroscopy; r.m.s.d., root mean square deviation; Ub, ubiquitin; UBA, ubiquitin-associated domain; UBB⁺¹, the frame shift mutant of ubiquitin B; UPS, ubiquitin-proteasome system; HSQC, heteronuclear single quantum correlation; TROSY, transverse relaxation optimized spectroscopy; DsRed, Discosoma sp. red fluorescent protein.
the absence of a residue corresponding to Gly76 in Ub; instead, UBB\(^{+1}\) serves as a scaffold for ligation of Ub molecules to produce polyUb that is anchored by UBB\(^{+1}\) (on the unaffected Lys-48 site). These molecules are reportedly refractory to the deubiquitinating enzyme system (12). Consequently, when UBB\(^{+1}\)-anchored polyUb is targeted to the 26 S proteasome, it acts as a functional antagonist, inhibiting the activity of the proteasome and leading to Aβ neurotoxicity (13, 14).

E2–25K is an unusual member of the E2 family in that it is competent to catalyze ubiquitin chain extension independent of E3 ligases (15). In addition to an E2 domain that is 22–48% identical to other human E2s, E2–25K also contains a UBA domain that is unique to this paralog (supplemental Fig. 1A). UBA domains appear to be generally involved in interactions with Ub, but the precise function of the E2–25K UBA domain is currently unclear, although evidence suggests it is important for polyubiquitylation activity. Moreover, a chimeric protein in which the UBA domain of E2–25K was fused to the E2 domain of yeast UBC4 showed no polyUb synthetic activity, suggesting that polyubiquitylation by E2–25K is dependent on the relative conformations of the E2 and UBA domains and their specific interactions (15). Interestingly, it was recently reported that an active site mutation, C92S or S86Y, or deletion of the UBA domain of E2–25K eliminated Aβ neurotoxicity (13). Based on these findings, it was proposed that UBB\(^{+1}\) interacts with the UBA domain of E2–25K and participates in the polyubiquitylation process that produces the UBB\(^{+1}\)-anchored polyUb chains. Details of the mechanism are not yet known, however.

In this report we present the three-dimensional structures of UBB\(^{+1}\), E2–25K, E2–25K/Ub, and E2–25K/UBB\(^{+1}\) determined by x-ray crystallography and NMR spectroscopy. We also analyzed in detail the interaction between E2–25K and UBB\(^{+1}\) and propose that the accumulation of UBB\(^{+1}\)-anchored polyUb reflects a direct interaction between the E2–25K UBA domain and UBB\(^{+1}\) and results in cell death due to proteasomal inhibition.

**EXPERIMENTAL PROCEDURES**

**Cloning of E2–25K, UBB\(^{+1}\), and Ub—**The cDNAs encoding E2–25K, UBB\(^{+1}\), and Ub were amplified by PCR using synthetic oligonucleotides and subcloned into the Escherichia coli expression vectors pET32a (Amersham Biosciences) and pET28b (Novagen) and the eukaryotic expression vector pcDNA3. The sense primers were designed to encode the tobacco etch virus protease recognition sequence (ENLYFQG). Residue-specific mutations of E2–25K (M172A, G173A, L198A) were accomplished using the PCR mutation method (16).

**Protein Purification and Sample Preparation—**All plasmid DNAs were transformed into E. coli strain BL21 (DE3), which was grown in LB or M9 minimal medium for 12 h at 25 °C after induction with 1 mM isopropyl-1-thio-β-d-thiogalactopyranoside. After the affinity chromatography, we removed the TRX-His tag (pET32a) or His tag (pET28b) by incubation with tobacco etch virus or thrombin protease, respectively, for 12 h at 25 °C. The purified proteins were then subjected to size exclusion chromatography on Superdex 75 (Amersham Biosciences) equilibrated with final buffer. For NMR experiments, \(^{15}N\)- or \(^{13}C/\(^{15}N\)-labeled proteins were purified from cells grown in M9 minimal medium containing \(^{15}NH_4Cl\) (Cambridge Isotope Laboratories Inc.) with or without d-[\(^{13}C_6\)]glucose (Cambridge Isotope Laboratories Inc.).

**Crystallization and Structure Determination—**E2–25K, Ub, and UBB\(^{+1}\) were concentrated to 20, 30, and 30 mg/ml, respectively, using a centriprep (Amicon). The buffer used for E2–25K and Ub crystallization contained 20 mM HEPES-NaOH (pH 7.5) and 150 mM NaCl. The buffer used for UBB\(^{+1}\) crystallization contained 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl. E2–25K crystals were grown at 18 °C in a 2-μl sitting drop containing equal volumes of protein solution and mother liquor (100 mM sodium cacodylate (pH 6.6), 18% (w/v) PEG 8000, and 100 mM sodium acetate). The purified E2–25K was mixed with Ub or UBB\(^{+1}\) at a 1:2 molar ratio. Crystals of E2–25K/Ub and E2–25K/UBB\(^{+1}\) complexes were grown in a 2-μl hanging drop containing equal volumes of the protein solution and mother liquor (E2–25K/Ub: 100 mM HEPES-NaOH (pH 7.5), 25% (w/v) PEG 3550, and 50 mM sodium fluoride; E2–25K/UBB\(^{+1}\): 100 mM Tris-HCl (pH 8.5) and 25% (w/v) PEG 3350). The crystals were cryoprotected in reservoir solution supplemented with 10% or 25% (v/v) glycerol. All the diffraction data were collected at 100 K. Diffraction data for E2–25K were collected at 2.4 Å resolution on a Rigaku FR-E generator equipped with a R-AXIS IV\(^{2+}\) detector, then processed and scaled using CRYSTAL- CLEAR (Rigaku). The structure was solved using the molecular replacement method with the program AMORE (17) using the coordinates of yeast UBC1 (PDB code 1TTE) as the search model. Diffraction data for the E2–25K/Ub and E2–25K/UBB\(^{+1}\) complexes were collected at 2.8 and 1.8 Å resolution, respectively, at beam line 4A at the Pohang Accelerator Laboratory, Korea, then processed and scaled using HKL2000 (HKL Research) (18). The structures of the two complexes were solved using the molecular replacement method with the program MOLREP (19) using the structures of E2–25K and Ub (PDB code 1UBQ) as the search models. Native and complex structures were subjected to many cycles of manual rebuilding using the program O (20) and were refined using the programs CNS (21) and REFMAC5 (22). The final structures were analyzed using PROCHECK (23). The statistics for the structure refinement are summarized in Table 1.

**NMR Experiments and Backbone Dynamics—**All NMR experiments were performed in a mixture of 90% H\(_2\)O and 10% \(^{3}H_2\)O or 99% \(^{2}H_2\)O NMR buffer (50 mM Na\(_2\)PO\(_4\), 100 mM NaCl, 2 mM DTT, and pH 7.0) at 298 K on a Bruker DRX 900 MHz equipped with a CryoProbe\(^{TM}\) system. The chemical shift in H was referenced directly to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, and \(^{13}C\) and \(^{15}N\) were referenced indirectly to H (24). All spectra were processed using XWINNMR (Bruker Biospin Corp.) and NMRpipe/NMRDraw software (25). Triple resonance experiments were executed for backbone assignment (26). The side-chain assignments were complicated by HCCH-TOCSY (two-dimensional total correlation spectroscopy) and \(^{13}C\)-edited NOESY (27). \(^{15}N\)-Edited NOESY-HSQC (τm = 120 ms) and \(^{13}C\)-edited NOESY-HSQC (τm = 120 ms) experiments were used for gathering structure con...
strains. To investigate the dynamic properties of UBB$^{+1}$, the heteronuclear $^1H,^15N$ NOEs were measured (28). The XNOE measurements were performed on 1024 × 128 complex points for $t_2 \times t_1$ dimensions with a 5-s recycle delay.

\textbf{NMR Structure Calculation of UBB$^{+1}$}—The solution structure of UBB$^{+1}$ was obtained by applying CYANA (29). Distance restraints and angle constraints were gathered from NOESY experiments and the TALOS program (30). NOE restraints consisting of 1514 unambiguous NOEs (843 short range NOEs ($I - j = 1$), 216 medium range NOEs ($1 \leq I - j < 5$), and 455 long range NOEs ($5 \leq I - j$) and 106 torsion angle restraints were used for structure calculations (Table 2). The final structures were analyzed using PROCHECK (23). With the exception of residue Asp-82 in the tail region (0.01%), all residues were located on the favored and allowed regions (99.99%) of the Ramachandran plot. PyMOL (Delano Scientific LLC, San Carlos, CA) was used with APBS and MOLMOL (31) to analyze and visualize the final structures.

\textbf{NMR Titration and Binding Affinity Measurements—TROSY-HSQC} (32) was used in titration experiments performed at various molar ratios of $^15N$-labeled proteins to unlabeled counter proteins. For E2–25K titration, the molar ratios of $^15N$-labeled E2–25K to UBB$^{+1}$ or Ub were 1:1, 1:2.5, 1:5, 1:7.5, and 1:10. For UBB$^{+1}$ or Ub titration, the molar ratios were 1:1, 1:2.5, and 1:5. Chemical shift perturbations were calculated using the equation $\Delta \delta_{AV} = \left( (\Delta \delta_{1H})^2 + (\Delta \delta_{15N}/5)^2 \right)^{1/2}$, where $\Delta \delta_{AV}$, $\Delta \delta_{1H}$, and $\Delta \delta_{15N}$ are the average, proton, and $^15N$ chemical shift changes, respectively. We determined the dissociation constant ($K_D$) from the NMR titration. After confirmation of complex saturation at a molar ratio of 1:10, we selected six isotherms (Met-172, Gly-173, Phe-174, Val-190, Thr-194, and Leu-198) and plotted the proton chemical shift changes based on the molar ratio. For nonlinear curve fitting (33), we used the equation $y(x) = 0.5b[(x + 1 + a) - (x + 1 + a)^2 - 4x]^{0.5}$, where $x$, $a$, and $b$ are the unlabeled UBB$^{+1}$/Ub/$^15N$-labeled E2–25K molar ratio, $K_D$(total $^15N$ labeled E2–25K concentration), and maximum proton chemical shift change ($\Delta \delta_{1H}$), respectively, and $y(x)$ is the $\Delta \delta_{1H}$ at a given $x$. All calculations were done using ORIGIN 7.5 software (OriginLab Corp.).

\textbf{In Vitro Pulldown Assays—Ub or UBB$^{+1}$ were cloned into pGEX-4T-1 vector (Amersham Biosciences), expressed in
E. coli BL21 (DE3) cells, and purified on glutathione-Sepharose. Wild-type or mutant E2–25K was incubated with GST alone, GST-Ub, or GST-UBB\textsuperscript{+1} in 1× PBS at a molar ratio of 1:1 for 3 h at 4°C in the presence of 50 μl of glutathione-Sepharose beads (Peptron). The bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by Western blotting using an anti-E2–25K antibody.

Cell Culture, DNA Transfection, and Cell Death Assays—B103 cells (rat neuroblastoma cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cells were transfected using Lipofectamine\textsuperscript{TM} reagent (Invitrogen) according to the manufacturer's protocol, after which their viability was assessed based on the morphology of GFP\textsuperscript{+} cells viewed under a fluorescence microscope (Leica DMRBE). We measured the accumulation green fluorescent protein fused to a short degron (GFPu) and Discosoma sp. red fluorescent protein (DsRed) as an index of intracellular proteasome activity in 293F cells overexpressing wild-type E2–25K or E2–25K mutants. Twenty-four hours after transfection of pDsRed plasmid and wild-type or mutant pGFPu-E2–25K plasmid into 293F cells, the cells were examined under a fluorescence microscope. 293F cells expressing only GFP (pcDNA3) were detected based on their red color, because the GFP was successfully removed by proteasome, only DsRed was detected. E2–25K transfecants were detected based on their yellow color, reflecting the combination of the green fluorescence from GFPu and red fluorescence from DsRed.

Proteasome Inhibition Assay—Inhibition of proteasome was examined as previously described by Song et al. (13). Briefly, pGFPu (an artificial proteasome substrate; 50 ng) together with each plasmid expressing E2–25K (500 ng) and pDsRed (50 ng) was transiently transfected into 293F cells. After 24 h the transfecants were examined under a fluorescence microscope to determine the relative numbers of GFP\textsuperscript{+} and DsRed\textsuperscript{−} cells.

Analysis of UBB\textsuperscript{+1}-anchored PolyUb Synthesis—To assay UBB\textsuperscript{+1} anchoring, the ubiquitylation activities of E2–25K and its mutants were measured based on an in vitro reaction run with UBB\textsuperscript{+1} (50 μM), Ub (50 μM), E1 (0.1 μM), E2–25K (1 μM), and ATP (5 mM). The 20-μl reaction mixtures containing wild-type or mutant E2–25K proteins were incubated for 4 h at 37°C and analyzed by Western blotting with rat polyclonal anti-UBB\textsuperscript{+1} antibody (2000:1), which was previously described by Song et al. (13).

RESULTS

Crystal Structure of E2–25K—Two molecules of E2–25K were observed in the asymmetric unit; the overall conformations of the two E2–25K proteins were almost identical, with a root mean square deviation (r.m.s.d.) of 0.77 Å for 196 C\textsubscript{α} atom pairs. The crystal structure of E2–25K revealed two well-defined domains, the E2 and ubiquitin associating (UBA) domains, that are linked by a short tether (154-GAPVSS-159) and tightly interact (Fig. 1A). The canonical E2-core-fold (residues 1–153) is defined by a β-sheet (β1-β4) with flanking helices (α1-α6). The UBA domain consists of a three-helix bundle (α7-α9) with a hydrophobic core formed by helix α7 (Leu-166 and Leu-169), helix α8 (Val-179 and Leu-183) and a loop connects helices α8 and α9 (Trp-188). This structure is similar to that of a recently described E2–25K M172A mutant (34); the r.m.s.d. between that structure and ours was 0.64 Å for 200 C\textsubscript{α} atom pairs.

The E2 and UBA domains interact with one another through both hydrophobic and electrostatic interactions. The interface is made predominantly by helices α5-α6 of the E2 domain and helices α7-α8 of the UBA domain (Fig. 1B). Tyr-162, Ile-180,
and Trp-188 of the UBA domain form a hydrophobic patch adjacent to residues Trp-80, Met-140, Leu-147, Trp-148, and Val-151 of the E2 domain (Fig. 1B). The hydrophobic interactions between the two domains are further stabilized by several hydrogen bonds (Tyr-152–Asn177, Trp-148—Ser-184, and Thr-144—Trp-188), and the 6-residue tether also contributes to the clustering of the two domains through both hydrophobic and electrostatic interactions. The active site is composed of the /H9252_4- and /H9251_5 loops. The Ub conjugating residue Cys-92 and the residue involved in activity regulation, Ser-86, are situated in close proximity to Asn-83 and Asp-127, respectively (Fig. 1C).

Crystal Structure of E2–25K/Ub Complex—To understand the structural basis of Ub recognition by E2–25K, we solved the structure of the E2–25K/Ub complex (Fig. 2A). The hydrophobic interactions between the two domains are further stabilized by several hydrogen bonds (Tyr-152—Asn177, Trp-148—Ser-184, and Thr-144—Trp-188), and the 6-residue tether also contributes to the clustering of the two domains through both hydrophobic and electrostatic interactions. The active site is composed of the β4-α3 and α4-α5 loops. The Ub conjugating residue Cys-92 and the residue involved in activity regulation, Ser-86, are situated in close proximity to Asn-83 and Asp-127, respectively (Fig. 1C).

Crystal Structure of E2–25K/Ub Complex—To understand the structural basis of Ub recognition by E2–25K, we solved the structure of the E2–25K/Ub complex (Fig. 2A). The structures of the Ub-complexed and apo forms of E2–25K were nearly identical with an r.m.s.d. of 0.77 Å for 196 Cα atom pairs. E2–25K binds to Ub mainly via residues in the α7-α8 loop and helix α9 and involves about 400 Å² of surface area (4.1% of total surface area) (Fig. 2B). That the surface of the intermolecular contact is relatively small is consistent with the low binding affinity (Kd = 400 μM) between E2–25K and Ub (35), as compared with other UBA domains whose affinities for Ub have been reported (Kd = 10–500 μM) (36). The UBA domain interacts with Ub mainly via the hydrophobic patches formed by Leu-8, Ile-44, His-68, and Val-70 of Ub and Met-172, Phe-174, Val-190, Thr-194, and Leu-198 of the UBA domain. Electrostatic interactions further stabilize the hydrophobic interactions; the backbone carbonyl group of Met-172 and the side chain of Glu-195 in the Ub interact with the backbone amide group of Gly-47 and the side chain of Arg-42 in Ub, respectively (Fig. 2B). The backbone NMR resonance assignments of
E2–25K were completed using data from TROSY-based triple resonance experiments (supplemental Fig. 2). The TROSY-HSQC spectra for E2–25K at various molar ratios in solution with Ub were consistent with the crystal structure and showed that residues involved in intermolecular interaction experience marked chemical shift changes upon Ub binding (Fig. 2C and supplemental Fig. 3A). The average \( K_d \) was 1.514 ± 0.342 mM. Sequence comparison of the E2–25K UBA domain with other UBA domains showed that the residues forming the hydrophobic patch that interact with Ub are well conserved (supplemental Fig. 1B).

To confirm the contribution made by critical residues in E2–25K to Ub binding, we carried out pulldown assays with various E2–25K mutants. In contrast to wild-type E2–25K, single mutations (M172A, F174A, V190A, T194A, and L198A), a double mutation (V190A/T194A), and a triple mutation (M172A/F174A/L198A) within the UBA domain all disrupted E2–25K ability to bind Ub, whereas a G173A mutation, which did not affect the hydrophobic interaction, had little effect on binding (Fig. 2D).

**Structure of the E2–25K/UBB Interaction**—The solution structure of UBB shows a well structured and defined region with a Ub sequence (M1-G75) and a C-terminal structure with an aberrant tail (Tyr-76—Gln-95) (Fig. 3A and supplemental Fig. 3B). When we separately superimposed the two regions using backbone atoms, the Ub region was well fitted with an r.m.s.d. of 0.54 ± 0.16 Å, whereas the tail region (G75-H88) had an r.m.s.d. of 1.86 ± 0.23 Å. This implies that the aberrant tail of UBB has a structure, although it is not well defined due to a lack of NOE information. Heteronuclear NOE data were consistent with the structural tendency of the tail region, *i.e.* the tail region had a heteronuclear NOE value of about 0.48 (Fig. 3B), which indicates the region has a structure that is independent of the Ub region. In addition, the two regions of UBB show distinctly different dynamic behaviors (supplemental Fig. 3C).

To better understand the structural basis of the interaction between UBB and E2–25K, we determined the crystal structure of the E2–25K/UBB complex. The overall structure of the complex was similar to that of the E2–25K/Ub complex; the r.m.s.d. between the two structures was 1.08 Å for 269 Cα atom pairs (Fig. 4, A and B). In addition, the r.m.s.d. between E2–25K in complex with Ub and UBB was 0.69 Å for 197 Cα atom pairs, whereas the r.m.s.d. between Ub and UBB in complex with E2–25K was 0.59 Å for 72 Cα atom pairs. Notably, the electron density for the main chain C-terminal residues (amino acids 73–75) was clearly observable in the E2–25K/UBB structure, whereas this region was highly disordered and invisible within the Ub complex. Among these residues, Leu-73 was highly ordered, and its side chain was clearly observable in the electron density map. Leu-73 is involved in the intermolecular interaction between E2–25K and UBB that enables the UBB molecule to tilt 15° with respect to Ub (Fig. 4C and supplemental Fig. 4).

To confirm the residue-specific interactions between E2–25K and UBB, we mutated E2–25K residues involved in UBB binding and assessed UBB binding in pulldown assays. Single mutations (M172A, F174A, V190A, T194A, and L198A), a double mutation (V190A/T194A), and a triple mutation (M172A/F174A/L198A) all significantly disrupted UBB binding, whereas the G173A mutation had little effect (Fig. 4D). Thus, UBB appears to interact with the E2–25K UBA domain via its hydrophobic residues in the same manner as Ub interacts with E2–25K (Fig. 4B).

We next carried out a set of NMR titration experiments to examine the mode of E2–25K binding to UBB in solution. Fig. 5A shows the chemical shift changes in E2–25K in the presence of UBB. Consistent with the crystal structures, most of the changes clearly seen upon Ub binding are in the UBA domain. However, at high concentration of UBB, small changes are also observed in the E2 domain, presumably due to nonspecific interactions involving the flexible tail. Met-172, Gly-173, Phe-174, Val-190, and Leu-198 in the UBA domain all showed chemical shift changes greater than 0.18 ppm, whereas Ala-171 and Thr-194 showed changes of 0.08–0.18 ppm (Fig. 5B).

In a set of similar experiments, we also examined the effect of E2–25K binding on UBB. Most changes elicited by E2–25K binding occurred in \( \beta3 \), \( \beta4 \), and the \( \alpha2-\beta3 \) loop (Fig. 5C), which
form the hydrophobic patch (Fig. 5D). This finding confirms that intermolecular contacts between E2–25K and UBB\(^{+1}\) in solution occur mainly between hydrophobic residues, as was observed in the crystal structure. In addition, Lys-48 and Leu-71 of UBB\(^{+1}\) showed larger chemical shift changes than were seen with Ub titration (Fig. 5C and supplemental Fig. 5C). The rigid C-terminal region (residues 73–75) observed in the crystal structure may contribute to the dramatic change in chemical shift for Leu-71, although the chemical shift changes for residues 73–75 themselves were little affected, probably because their perturbation was not sufficient to induce significant transition of the backbone amide proton environment.

We next used a nonlinear curve-fitting method to determine the \(K_d\) for the binding of E2–25K and UBB\(^{+1}\) derived from the NMR titrations. Based on the binding isotherms, we calculated the plots of \(\Delta\delta_{HH}\) versus molar ratio for residues Met-172, Gly-173, Phe-174, Val-190, Thr-194, and Leu-198 (supplemental Fig. 3D and Table 1). The average \(K_d\) for E2–25K binding to UBB\(^{+1}\) was 0.939 ± 0.218 mM, whereas the \(K_d\) for E2–25K binding to Ub was 1.514 ± 0.342 mM.
The greater apparent affinity of E2–25K for UBB⁺¹ is consistent with the larger area (440 Å²) of the E2–25K-UBB⁺¹ interface, as compared with the E2–25K/Ub interface (400 Å²).

To evaluate the importance of the Ub/UBB⁺¹ binding site in the assembly of UBB⁺¹-anchored polyUb, we carried out polyubiquitylation assays with E2–25K mutants in the presence of UBB⁺¹ (Fig. 6A). An E2–25K deletion mutant lacking the UBA domain showed almost no UBB⁺¹-anchored products, whereas wild-type E2–25K readily formed UBB⁺¹-anchored polyUb (Ub₁-UBB⁺¹ and Ub₁-Ub₂-UBB⁺¹). Most of the E2–25K substitution mutants studied also showed a diminished ability to catalyze the synthesis of UBB⁺¹-anchored polyUb, indicating that the interaction between the UBA domain and UBB⁺¹ is important for synthesis of UBB⁺¹-anchored polyUb.

FIGURE 5. NMR mapping of E2–25K/UBB⁺¹ interactions in solution. A, ¹⁵N-labeled E2–25K was titrated with UBB⁺¹. The bar diagram shows the chemical shift changes at a E2–25K/UBB⁺¹ molar ratio of 1:5. The residues showing chemical shift changes above 0.08 ppm (Δδ) are labeled and classified as green (0.08 ppm ≤ Δδ < 0.18 ppm), blue (0.18 ppm ≤ Δδ < 0.3 ppm), and brown (0.3 ppm ≤ Δδ < 0.5 ppm). B, UBB⁺¹ binding sites are indicated by different colors based on chemical shift changes (Δδ) as described in A. In the magnification of the UBA domain, residues interacting with UBB⁺¹ are colored based on the chemical shift changes and labeled based on the surface charge model in the inset box. C, ¹⁵N-labeled UBB⁺¹ was titrated with E2–25K. The bar diagram shows the chemical shift changes at a UBB⁺¹:E2–25K molar ratio of 1:5. The residues showing chemical shift changes greater than 0.1 ppm (Δδ) are labeled and classified as green (0.12 ppm ≤ Δδ < 0.24 ppm), blue (0.24 ppm ≤ Δδ < 0.48 ppm), and red (0.48 ppm ≤ Δδ < 1 ppm). Red stars indicate the residues lost due to peak broadening. D, E2–25K-binding residues are presented in the surface model of UBB⁺¹. The different colors are defined as in C.
Structural Basis of E2–25K and UBB\textsuperscript{+1} Interaction

![Image of a diagram](image.png)

**FIGURE 6. Proteasome inhibition and neurotoxicity assays.** A, polyubiquitylation reactions using Ub and UBB\textsuperscript{+1} mixtures were run with Ub (50 μM), UBB\textsuperscript{+1} (50 μM), E1 (0.1 μM), and E2–25K wild type or its mutants (1 μM) (with ATP (5 mM) for 4 h at 37 °C). PolyUb chains anchored by UBB\textsuperscript{+1} were analyzed by Western blotting using a rat polyclonal anti-UBB\textsuperscript{+1} antibody. B, the relative GFPu levels in B103 cells transfected with empty vector, E2–25K, or one of the E2–25K mutants were determined by fluorescence microscopy. Bars depict the means ± S.D. (n = 4). C, B103 cells were transfected with empty vector (pcDNA3), wild-type E2–25K, and the most effective E2–25K mutants (M172A/F174A/L198A triple mutant and V190A/T194A double mutant), after which GFPu accumulation was assessed by examining the cells under a fluorescence microscope. GFPu and DsRed were used to detect intracellular proteasome activity. Red and green indicate basal protein expression and GFPu accumulation in the cytosol, respectively. Yellow indicates overlap of the red and green signals. D, B103 cells were transfected with empty vector (pcDNA3), E2–25K, or one of the E2–25K mutants for 48 h, after which cell viability was assessed based on the morphology of DsRed\textsuperscript{+} cells examined under a fluorescence microscope. Bars depict means ± S.D. (n = 4).

polyUb, showed significantly lower levels of GFP accumulation (Fig. 6B). Collectively, these data strongly suggest that the E2–25K/UBB\textsuperscript{+1} interaction plays a key role in mediating UPS impairment through the formation of UBB\textsuperscript{+1}-anchored polyUb.

We previously reported that proteasome inhibition by UBB\textsuperscript{+1}-anchored polyUb is associated with neurotoxic activity in transfected neuronal cells (13). Given the structure of E2–25K/UBB\textsuperscript{+1}, we surmised that the UBA domain of E2–25K is responsible for controlling the ubiquitylation of UBB\textsuperscript{+1}, which is a major factor in proteasome inhibition and UBB\textsuperscript{+1}-induced neurotoxicity. To confirm the role of the E2–25K/UBB\textsuperscript{+1} interaction in neurotoxicity, we carried out cell death assays using various E2–25K mutants with B103 neuronal cells. Mutations within the UBA domain led to reductions in cell death, as compared with wild-type E2–25K (Fig. 6, C and D). Correlations between the reduction in cell death and the positions of the mutated residues are presented in Fig. 6, C and D. They show that the critical residues whose mutation reduces the incidence of cell death are located within the hydrophobic patch formed by helices α7 and α9. Taken together these findings suggest that the interaction between UBB\textsuperscript{+1} and the UBA domain of E2–25K and the resultant formation of UBB\textsuperscript{+1}-anchored polyUb are directly responsible for the neuronal cell death caused by proteasome inhibition.

**DISCUSSION**

E2–25K is known to mediate Aβ neurotoxicity by promoting the inhibition of proteasome activity, altering the ER stress response, and activating caspase-12-dependent cell death (13, 38, 39). In addition, accumulation of polyUb-anchored UBB\textsuperscript{+1}, the synthesis of which is in part catalyzed by E2–25K, directly inhibits proteasome activity. Notably, we observed that active site mutations (C92S or S86Y) or deletion of the E2–25K UBA domain eliminated Aβ neurotoxicity (13), and we propose that by interacting with UBB\textsuperscript{+1} via the UBA domain, E2–25K contributes to the formation of UBB\textsuperscript{+1}-anchored polyubiquitin chains. To investigate the structural and functional role of the UBA domain in Ub/UBB\textsuperscript{+1} binding, UBB\textsuperscript{+1}-anchored polyUb synthesis, and proteasome inhibition-mediated Aβ neurotoxicity, we used x-ray crystallography and NMR spectroscopy to determine the three-dimensional structures of UBB\textsuperscript{+1}, E2–25K, and the E2–25K/Ub and E2–25K/UBB\textsuperscript{+1} complexes.

**Rigid Interaction between the E2 and UBA Domains**—Among E2 proteins, E2–25K and its yeast homolog, UBC1, are unique in that they contain a C-terminal UBA domain in addition to an E2 domain. The E2 and UBA domains of UBC1 are connected by a flexible, 22-residue linker and are presumably independent of one another (40, 41). By contrast, the domains in E2–25K are linked by a short, six-residue tether and extensively interact via hydrophobic residues, resulting in a single globular topology. These structural differences likely affect the respective polyUb chain-building properties of E2–25K and UBC1. For instance, whereas the UBC1 UBA domain is involved in autoubiquitylation at Lys-93, E2–25K, in the absence of E3 enzymes, is responsible for the synthesis of polyUb chains that are not anchored by substrates (42, 43). Haldeman et al. (43) attempted to define the function of the E2–25K UBA domain by making a chimeric protein in which the E2–25K UBA domain and its short tether were fused to the E2 domain of yeast UBC4, which has no ability...
Structural Basis of E2–25K and UBB + 1 Interaction

Ubb/UBB + 1 Interaction with the E2–25K UBA Domain and Polyubiquitylation—The UBA domain of E2–25K is composed of three α helices. The third helix and the loop between the first two (MGF motif) mediate the interaction with Ub in four UBA-Ub complexes (UQ1, PDB code 2Y6; Ede1m PDB code 2G3Q; Dsk2p, PDB code 1WRI; EDD, PDB code 1QHO) (supplemental Fig. 6 and Refs. 45–48). Moreover, substitution of the Met or Phe residues in the MGF motif or the Leu residue in α3 disrupts that interaction. The UBA domain of E2–25K binds Ub mainly via the α7-α8 loop (corresponds to the α1-α2 loop in other UBA structures) and helix α9 (corresponds to α3). In addition to the well studied Met-172 and Phe-174 residues in the MGF motif and the Leu-198 residue in α9, mutation of Val-190 and/or Thr-194 in α9 dramatically disrupted Ub binding, indicating helix α9 is as important as the MGF motif for the E2–25K UBA-Ub interaction (supplemental Fig. 5B).

We have shown that Ub and UBB + 1 interact with the E2–25K UBA domain in similar fashion. Within the E2–25K/UBB + 1 complex, however, three additional residues were visible in the Ub region (residues 73–75, which were invisible in E2–25K/Ub structure), resulting in an increase in the surface area of the E2–25K/Ub + 1 interface (~440 Å2), as compared with the E2–25K/Ub interface (~400 Å2) (supplemental Fig. 4). Consistent with the greater area of interaction, UBB + 1 showed a 1.5-fold greater affinity for E2–25K than Ub. In particular, the β4-strand of UBB + 1 is stabilized by the tail region, resulting in an increase in binding affinity.

It is known that UBB + 1 expressed under basal conditions can be removed by the UPS but that up-regulated expression of UBB + 1 leads to inhibition of the 26S proteasome and, subsequently, cell death (11). Inhibition of the 26S proteasome is also dependent on formation of UBB + 1-anchored polyUb chains (37). We showed that E2–25K mutation that disrupted UBB + 1 binding diminished the E2–25K ability to synthesize UBB + 1-anchored polyUb (Fig. 6A). Thus, the E2–25K UBA domain, especially the MGF motif and α9, is critical for the synthesis of polyUb-anchored by UBB + 1.

E2–25K UBA-dependent Proteasome Inhibition and Neurotoxicity—UBB + 1 accumulates in the brains of Alzheimer disease patients, and UBB + 1-anchored polyUb potently inhibits proteasome activity, which leads to neurotoxicity (3). Notably, when transfected into neuronal cells, a UBB + 1 K48R mutant exhibited no ability to inhibit proteasome and no neurotoxicity (12, 13, 37), suggesting that inhibition of proteasome activity by UBB + 1-anchored polyUb synthesized by E2–25K is associated with the observed neurotoxicity. In this report we verified that

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