所であると予想される。

PINK1はUnokiらにより癌関連遺伝子であるPTENを活性化した際に高発現する遺伝子として単離された。PINK1遺伝子は581のアミノ酸からなり，マウスと95%の相同性をもつことから種間を共通して保存されている重要な蛋白であると予想される。構造上，最初の34のアミノ酸残基にミトコンドリアへの移行シグナルを持ち，後半の大部分をカルモジュリンファミリーのセリン/トレオニンキナーゼドメインが占める。PARK8のLRRK2同様リン酸化酵素に関連性が指摘されておりPDの共通機序にミトコンドリア機能，酸化ストレス，蛋白分解系，そしてリン酸化が共通機序として考えられる。

これまでにイタリア5家系，日本4家系，スペイン，フィリピン，台湾，イスラエルそれぞれ1家系ずつの報告がある。まだ症例数が少なく，人種間における差異，頻度など明確な点は不明であるが，アジアでは比較的頻度が高い傾向がありparkin陰性例の約10%がPINK1遺伝子異常をもつと考えられる。その臨床型は，発症年齢は平均32±7.87才，罹患期間平均17.5年，l-dopaが奏功し，Park2に比し発症時のジストニア，睡眠効果，日内変動，腱反射亢進を呈する症例は少ない傾向がある。精神症状は稀ではあるが，一部の患者に発症前に統合失調症様症状，うつ状態を認めている。若年の発症で孤発型に類似した臨床症状であればPINK1遺伝子異常を検索する必要があると思われる。イタリアのホモ接合体変異の家系において18F-dopaの線条体での取り込み低下が認められていが，心筋シンチ，SPECTの報告は未だない。剖検例もなく，Lewy小体の有無は不明である。

4．その他の遺伝子異常

ADの家系において認められた原因遺伝子にUCH-L1とNurr1があるが，追試の報告はなく，非常に稀な変異である可能性がある。UCH-L1はユビキチンリガーゼ活性を持つとの報告もあり，UPSでの機能解明が期待される。

5．PDにおける選択的細胞変性へのプロセス

家族性パーキンソニズムの原因遺伝子産物は酸化ストレス，UPS機能低下，ミトコンドリア機能低下，リン酸化との関連性が指摘されている(表2)。これら因子は同時にPD発症の誘因としても注目されており，FPDとPDには共通したメカニズムが存在していると言える。また多くの遺伝子産物がLewy小体の構成成分であることからもその機能破綻が黒質ドパミン神経細胞死に関与している可能性がある。

おわりに

上記の既存の遺伝子異常を認めないFPDの患者も数多く存在する。事実，ARPDであっても未だ40%のFPDは原因遺伝子が不明である。現在我々の研究グループでも精力的に解析が進められている。ゲノムワイドな連鎖解析，その候補領域の検討も行われており，新規遺伝子座あるいは感受性遺伝子の発見も待たれる。またSNPによる薬剤の個体での反応の差異，原因遺伝子に対応した薬剤の選択法など，個々患者に対応した遺伝子レベルでの治療方針，つまりオーダーメイド医療の確立もそう遠くない未来にくるかもしれない。

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DDB2, the xeroderma pigmentosum group E gene product, is directly ubiquitylated by Cullin 4A-based ubiquitin ligase complex

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Abstract

Xeroderma pigmentosum (XP) is a genetic disease characterized by hypersensitivity to UV irradiation and high incidence of skin cancer caused by inherited defects in DNA repair. Mutational malfunction of damaged-DNA binding protein 2 (DDB2) causes the XP complementation group E (XP-E). DDB2 together with DDB1 comprises a heterodimer called DDB complex, which is involved in damaged-DNA binding and nucleotide excision repair. Interestingly, by screening for a cellular protein(s) that interacts with Cullin 4A (Cul4A), a key component of the ubiquitin ligase complex, we identified DDB1. Immunoprecipitation confirmed that Cul4A interacts with DDB1 and also associates with DDB2. To date, it has been reported that DDB2 is rapidly degraded after UV irradiation and that overproduction of Cul4A stimulates the ubiquitylation of DDB2 in the cells. However, as biochemical analysis using pure Cul4A-containing E3 is missing, it is still unknown whether the Cul4A complex directly ubiquitylates DDB2 or not. We thus purified the Cul4A-containing E3 complex to near homogeneity and attempted to ubiquitylate DDB2 in vitro. The ubiquitylation of DDB2 was reconstituted using this pure E3 complex, indicating that DDB–Cul4A E3 complex in itself can ubiquitylate DDB2 directly. We also showed that an amino acid substitution, K244E, in DDB2 derived from a XP-E patient did not affect its ubiquitylation.

Keywords: Nucleotide excision repair; E3; Ubiquitin; DDB1; DDB2; Cullin 4A

1. Introduction

Several proteins that bind specifically to ultraviolet (UV) irradiation damaged-DNA have been discovered by electrophoretic mobility shift assay or filter-binding assay since 1970s [1]. Previous studies that have characterized the damaged-DNA binding (DDB) protein indicated that the minimal DDB complex is a heterodimer comprised of a 127 kDa DDB1 subunit and 48 kDa DDB2 subunit. The binding activity of damaged DNA is thought to reside in this heterodimeric complex (for reviews, see [2,3]).

Xeroderma pigmentosum (XP) is a rare genetic disease characterized by clinical and cellular hypersensitivity to UV radiation and high incidence of skin cancer [4]. Cells from XP patients show defective repair of DNA damage that had been induced by UV or chemical agents, and tendency for skin carcinogenesis. In 1988, Chu and Chang [5] reported that cells from XP complementation group E (XP-E) individuals (GM02415/XP2RO) lacked this damaged-DNA binding activity, suggesting that DDB is functionally involved in the XP-E disease. This is also true for some other alleles of XP-E

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patients [6]. Further evidence for the involvement came from microinjection experiments indicating that the purified DDB complex complements the XP-E cells' defect [7,8]. Other studies demonstrated that ectopic expression of human *DDB2* enhanced DNA repair in Chinese hamster V79 cells, which rarely express endogenous rodent *DDB2* [9]. Soon after the identification of *DDB1* and *DDB2* genes, Nichols et al. [10] revealed that *DDB2* was in fact mutated in XP-E cells lacking DDB activity. However, the molecular basis of the XP-E phenotype was ambiguous, because several groups found that cells from other patients with XP-E had normal levels of DDB activity (DDB+) and possessed no mutation in *DDB2* gene (reviewed in [2,3]). This discrepancy was puzzling until recently. Based on a thorough analysis, however, it was found that some DDB+ cell lines were mistakenly assigned to XP-E, and now it appears that all known authentic cases of XP-E are caused by *DDB2* mutations [11,12].

In eukaryotic cells, selective protein degradation is largely mediated by the ubiquitin/proteasome system. When ubiquitin is attached to the target protein by the ubiquitylation machineries, the proteasome recognizes the poly-ubiquitylated substrate to be degraded. This ubiquitin conjugating system requires the cascade reaction of three enzymes, namely E1, a ubiquitin-activating enzyme, E2, a ubiquitin-conjugating enzyme, and E3, a ubiquitin ligase. In 1999, Shiyanov et al. [13] reported that Cullin 4A (Cul4A) associates with the DDB complex. The cullin family of proteins compose a multimeric E3 complex. Cullin 1, which is the most well characterized cullin, serves as a rigid scaffold of its E3 complex and catalyses ubiquitylation through appropriate positioning of E2 and the substrate [14]. Other cullin family proteins including Cul4A are believed to function as well. The interaction between Cul4A and DDB1 was also demonstrated by several other groups recently ([15–20] and this work). These results, together with the rapid degradation of DDB2 after UV irradiation [21,22], suggest the involvement of Cullin 4A in DDB2 ubiquitylation and degradation. Strikingly, over-production of Cul4A stimulates the ubiquitylation of DDB2 [15,16]. However, since the latter studies did not show biochemical evidence of Cul4A involvement in the ubiquitylation of DDB2, it is still unknown whether the Cul4A-containing E3 complex in itself directly ubiquitylates DDB2 or not. To further investigate the mode of this ubiquitylation, an in vitro reconstitution by biochemical approach is obviously required. Here, we show that DDB2 can be ubiquitylated directly by the purified DDB–Cul4A E3 complex in a reconstitution in vitro experiment.

2. Materials and methods

2.1. Protein identification by LC–MS/MS analysis

The Cullin 4A-associated complexes were digested with *Achromobacter* protease-I and the resulting peptides were analyzed using a nanoscale LC–MS/MS system as described previously [23,24]. The peptide mixture was applied to a Mightysil-PR-18 (1 μm particle, Kanto Chemicals, Tokyo, Japan) frit-less column (45 mm × 0.150 mm i.d.) and separated using a 0–40% gradient of acetonitrile containing 0.1% formic acid over 30 min at a flow rate of 50 nl/min. Eluted peptides were sprayed directly into a quadruple time-of-flight hybrid mass spectrometer (Q-T of *Ultima*, Micromass, Manchester, UK). MS and MS/MS spectra were obtained in data-dependent mode. Up to four precursor ions above an intensity threshold of 10 counts/s were selected for MSMS analyses from each survey scan. All MS/MS spectra were searched for protein sequences of Swiss Prot and RefSeq (NCBI) using batch processes of Mascot software package (Matrix Science, London, UK).

2.2. Cell culture condition

High-Five insect cells were maintained as an adherent culture in Grace insect media (Invitrogen, Carlsbad, CA, USA) supplemented with 8% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin-streptomycin (Invitrogen). ts41 cells established from Chinese hamster [25] were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum and 1% penicillin–streptomycin under 5% CO_2 condition at 34 °C.

2.3. Immunoprecipitation experiment

To express DDB1, DDB2 and cullin family proteins, all plasmids were constructed from pcDNA3 or pcDNA3.1 plasmid (Invitrogen). Additional details of the plasmid construction processes will be provided upon request. Mammalian ts41 cells at 48 h after DNA transfection were harvested, washed by phosphate-buffer saline (PBS) and lysed with buffer A containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 and 10% glycerol. After removal of the debris by centrifugation, anti-Flag antibody (M2)-conjugated agarose (Sigma) was added to the lysate and the mixture was incubated at 4 °C for 2 h under constant rotation. After extensive washing of immunoprecipitates with buffer A, binding proteins were eluted with sodium dodecyl sulphate (SDS)-containing buffer and boiled at 95 °C for 5 min. The eluate was subjected to immunoblotting using anti-Flag (M2; Sigma), anti-Myc (Santa Cruz, Delaware, CA, USA), anti-Cul4A (our laboratory collection) and anti-DDB1 antibodies (Zymed, San Francisco, CA, USA).

2.4. Protein purification

To overproduce His-DDB1, Flag-DDB2, Cullin 4A-HA and T7-Rbx1 proteins in insect cells, the tagged full-length cDNAs were inserted into pFastBac donor plasmid (Invitrogen). Additional details of the plasmid construction processes can be provided upon request. Subsequent production of baculovirus particles was carried out according to the protocol provided by the manufacturer. Baculovirus particles for His-

DDB1 and Flag-DDB2 were used to simultaneously infect High-Five cells, as well as viruses for Cullin4A-HA and T7-Rbx1. Insect cells were incubated for 48 h after infection, washed using PBS at 4 °C and then harvested by centrifugation. The cell extract was collected using buffer B containing 20 mM Tris–HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 100 μM $ZnSO_4$, 10 mM 2-mercaptoethanol, 6% glycerol and a protease inhibitor mixture without ethylenediaminetetraacetic acid (EDTA) (Roche, Mannheim, Germany). After centrifugation, the cell lysates were mixed together and incubated at 4 °C for 5 h with occasional gentle mixing. For initial purification, the cell lysate was loaded on a single-stranded DNA cellulose (Sigma) column equilibrated with buffer B. The column was then washed with buffer B containing 0.3 M NaCl followed by elution with buffer B containing 0.7 M NaCl. The eluted fraction was subsequently purified with nickel-chelating agarose (Qiagen, Stanford, CA, USA) pre-equilibrated with buffer B and eluted by 120 mM imidazole. This purified complex was further separated on a glycerol gradient sedimentation, which was carried out through a 10–40% glycerol gradient in 25 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol (DTT) and 2 mM ATP for 22 h at 25,000 rpm ultracentrifugation. Fractions of 1 ml were collected from the top of the gradient and subjected to silver staining and immunoblotting.

To purify DDB2 (K244E)-containing complex, cell lysates containing His-DDB1, Flag-DDB2 (K244E), Cullin 4A-HA and T7-Rbx1 proteins in buffer B were collected as mentioned above. The DDB2 (K244E) complex was roughly purified with nickel-chelating agarose (Qiagen) pre-equilibrated with buffer B and eluted by 100 mM imidazole. Obtained fractions were then loaded onto HiTrap Heparin HP column (Amersham Biosciences, Piscataway, NJ, USA), washed with buffer C [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 100 μM $ZnSO_4$, 1 mM DTT, 4.5% glycerol and protease inhibitor mixture without EDTA (Roche)], and eluted with a 0.15–0.75 M NaCl gradient in buffer C. The DDB2 (K244E)-containing complex was eluted around 0.5 M NaCl and was subjected to dialysis with buffer D containing 20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 100 μM $ZnSO_4$, and 1 mM DTT. A protein complex containing wild-type DDB2 was simultaneously isolated by the same method and used as a control.

To collect the authentic DDB–Cul4A complex from mammalian cells, HeLa cells stably expressing N-terminally FLAG-HA-tagged DDB2 were used. The genuine DDB–Cul4A complex was immunoprecipitated with anti-FLAG antibody followed by anti-HA antibody as described previously [17]. The eluates were further purified by Mini Q (Amersham Biosciences) column chromatography instead of glycerol density gradient centrifugation.

2.5. *In vitro ubiquitylation assay*

The ubiquitylation assay was essentially performed as described previously [26,27]. Briefly, the purified DDB–Cul4A complex was incubated in 25 mM Tris–HCl, pH 7.5, 1 mM DTT, 25 μM MG132 (Peptide Inc., Osaka, Japan), 5 mM $MgCl_2$, 100 μM $ZnSO_4$, 2 mM ATP, 50 μg of ubiquitin (Sigma)/ml, 2 μg of E1/ml and 70 μg of various E2-expressing *Escherichia coli* lysate/ml at 32 °C for 2 h and subjected to immunoblotting with anti-His (penta-His antibody; Qiagen, Stanford, CA, USA), anti-HA (HA.11, Berkeley Antibody Company, Berkeley, CA, USA), anti-Flag (M2; Sigma) and anti-T7 (Novagen, Madison, WI, USA) antibodies. In some cases, GST-ubiquitin was used instead of native ubiquitin.

3. Results

3.1. *DDB complex physically interacts with Cullin 4A*

To explore the molecular function of Cullin 4A, we examined the cellular partner(s) that interact with Cul4A in cells. A thorough analysis of human EST and genome sequences showed that the registered human Cul4A sequence (659 amino acid protein [28]) lacks its N-terminal 100 amino acid residues and thus the full-length Cul4A was obtained by PCR-assisted cDNA cloning and used hereafter. The complete nucleotide sequence of full-length Cul4A has been registered under accession number AB178950.

Flag-tagged Cul4A was expressed in HEK293 cells followed by immunoprecipitation by anti-Flag antibody. The immunoprecipitates were eluted with a Flag peptide and then digested with Lys-C endopeptides (A. protease I) and the cleaved fragments were directly analyzed using a highly sensitive "direct nano-flow LC–MS/MS" system (for detail, see Section 2). Following database search, a dozen of peptides were assigned to MS/MS spectra obtained from four nano-LC–MS/MS analyses for the Flag-Cul4A-associated complexes and DDB1 was identified as one of the Cul4A-interacting proteins.

To confirm the interaction between Cul4A and DDB1, we performed immunoprecipitation experiment. Plasmids carrying Flag-tagged cullin family proteins (Cul1, 2, 3, 4A, 4B and 5) and myc-tagged DDB1 were concurrently transfected into ts41 cells. Extracts of the transfected or mock-transfected cells were subjected to immunoprecipitation using anti-Flag antibody followed by immunoblotting with anti-DDB1 antibody. As shown in Fig. 1A, Cul4A significantly interacted with DDB1. Cul1 also bound DDB1 weakly, whereas the other Cullins tested did not interact with DDB1. We next examined whether DDB2 also associates with Cul4A, because DDB1 and DDB2 are part of the DDB complex. Plasmids carrying 6myc-tagged cullin family proteins were transfected into ts41 cells along with a plasmid harboring Flag-tagged DDB2. Each extract was then subjected to immunoprecipitation using anti-Flag antibody and immunoblotting with anti-myc antibody. Consistent with the above results, DDB2 also interacted strongly with Cul4A and weakly with Cul1 and Cul4B (Fig. 1B). DDB2 did not bind with other cullin fam-

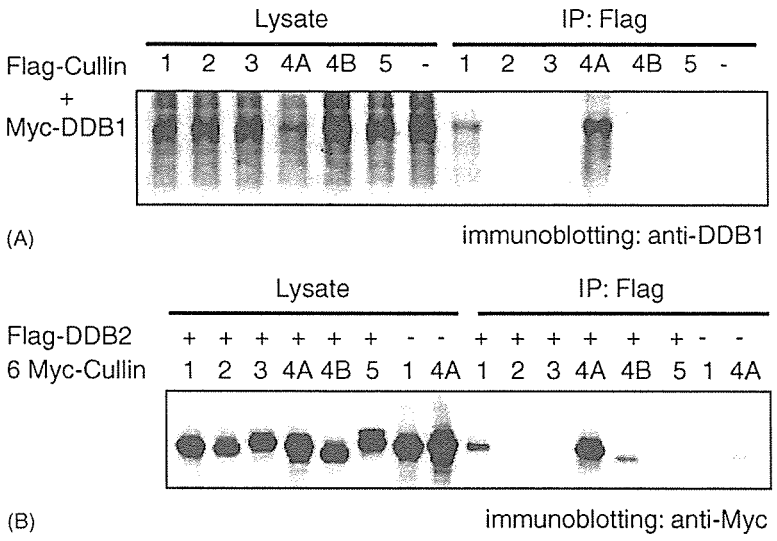

Fig. 1. DDB complex interacts with Cullin 4A. (A) Cul4A interacts with DDB1. Flag-tagged cullin family proteins and Myc-tagged DDB1 were simultaneously transfected into ts41 cells. After immunoprecipitation (IP) by anti-Flag antibody, the resulting immunoprecipitates were subjected to immunoblotting using anti-DDB1 antibody. (B) Cul4A also associates with DDB2. IP was similarly performed using FLAG-tagged DDB2 and Myc-tagged cullin family protein concurrently transfected into ts41 cells. After IP by anti-FLAG antibody, the resulting immunoprecipitates were analyzed using anti-Myc antibody.

ily proteins (Cul2, 3 and 5) examined. These results showed that DDB complex preferentially interacts with Cul4A, as reported previously [16].

3.2. Purification of DDB–Cul4A complex

We next attempted to purify DDB–Cul4A E3 complex using baculovirus expression system to perform biochemical experiments. Flag tag was fused to DDB2 at its N-terminus to facilitate its detection. This Flag-tagged DDB2 is thought to be functional because recent studies showed that ectopic expression of Flag-DDB2 enhanced DNA repair in Chinese hamster V79 cells [9], and purified Flag-DDB2 protein could restore damaged-DNA binding activity in extracts of XP-E patient cells [12]. DDB complex has been purified previously using DNA affinity column [13] and we also used DNA cellulose for initial purification of this complex. His6-tagged DDB1 and Flag-tagged DDB2 were simultaneously expressed in High-Five insect cells by the baculovirus induction system. Cul4A-HA and T7-Rbx1 were expressed concurrently as well. Each cell lysate was mixed and the resulting protein complex was purified by sequential column chromatography on single-stranded DNA cellulose, nickel-chelating agarose and subsequent 10–40% glycerol gradient by ultracentrifugation. The E3 complex comprised of DDB1, DDB2, Cul4A and Rbx1 was collected to near homogeneity as a peak fraction of glycerol gradient as shown in Fig. 2. Note that several other proteins were also detected in the final preparation (for example, a typical protein is shown by an asterisk in Fig. 2). However, since the peak fraction of such protein was inconsistent with that of the E3 complex in the glycerol gradient (data not shown), we think the protein is a contaminant derived from insect cells or a degradation product of the expressed protein, rather than a protein physiologically associated with the E3 complex.

3.3. DDB2 is ubiquitylated by purified Cul4A complex

Using this purified complex, we next tried to reconstitute the ubiquitylation of DDB2 to check whether DDB–Cul4A complex per se can ubiquitylate DDB2. Since E3 generally requires specific E2 to mediate ubiquitylation, we tested eight different E2 enzymes (E2-20k, E2-25k, Ubc3, Ubc4, UbcH5a, UbcH5c, Ubc7 and Ubc8). Slower-migrating ladders derived from auto-ubiquitylation of Cul4A (see below) were observed only from the reaction with Ubc4, UbcH5a and UbcH5c, whereas the other E2 enzymes tested did not support this modification (Fig. 3A). We thus used UbcH5 family as a source of E2 in the following experiments. Purified DDB–Cul4A complex was incubated with ATP, ubiquitin, E1 and UbcH5a, and subjected to immunoblotting with the antibody for each component. As expected, ladders derived from the auto-ubiquitylation of Cul4A were observed (Fig. 3B, single asterisk in the middle panel). Moreover, apparent high molecular-mass ladders were evident when DDB2 was detected using the anti-Flag antibody (Fig. 3B, single asterisk in the left panel). In order to demonstrate that this modification was due to ubiquitylation, we repeated the ubiquitylation assay in the presence or absence of ubiquitin. The slower migrating ladders were not detected without ubiquitin, and the addition of GST-ubiquitin instead of native ubiquitin resulted in the appearance of larger molecular-mass bands (Fig. 3B, double asterisks), indicating that this modification indeed is ubiquitylation. In the case of DDB1, a single high-molecular band also emerged after in vitro ubiquitylation (Fig. 3B, right panel). However, this ubiquitylation sig-

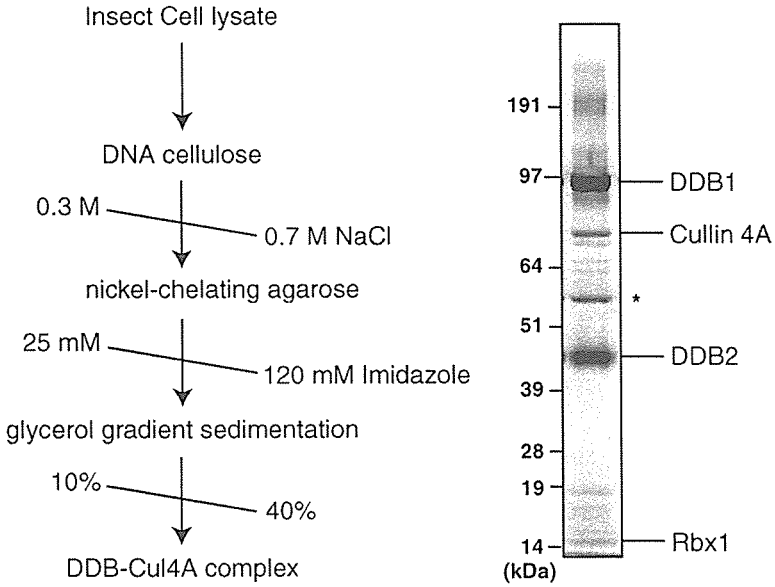

Fig. 2. Purification of the baculovirus-expressed DDB–Cul4A complex. The DDB–Cul4A complex was purified by sequential column chromatography and subsequently separated onto a 10–40% glycerol gradient by ultracentrifugation. The peak fraction of DDB–Cul4A complex was resolved by SDS-PAGE and visualized by silver staining. Asterisk shows the contaminant protein (see Section 3).

nal of DDB1 was fainter than that of Cul4A and DDB2 (see Section 4).

To further investigate the biochemical characteristics of DDB–Cul4A complex, we next purified it under more physiological conditions. HeLa cells stably expressing FLAG-HA-tagged DDB2 [17] were used to collect E3 complex. The DDB2-containing complex was immunoprecipitated with anti-FLAG antibody followed by anti-HA antibody as described previously [17] and the eluates were further purified by Mini Q column chromatography. The authentic E3 complex, comprised of DDB1, DDB2, Cul4A and Rbx1, was purified to almost homogeneity (Fig. 4A). When this complex was incubated with ATP, ubiquitin, E1 and UbcH5a, apparent high molecular-mass ladders derived from the ubiquity-

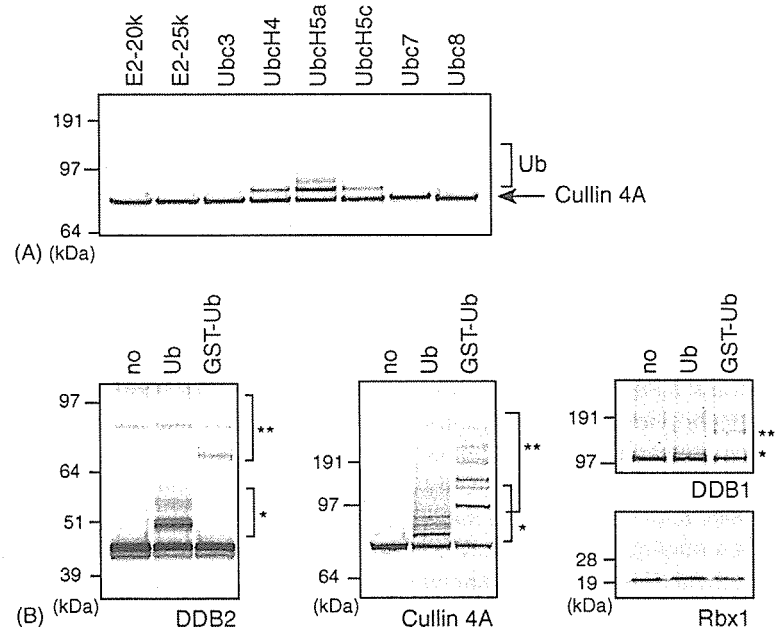

Fig. 3. In vitro reconstitution of DDB2 ubiquitylation. (A) The DDB–Cul4A E3 complex cooperates with Ubc4 and UbcH5 subfamily of E2 enzymes. Purified DDB–Cul4A E3 was incubated with the indicated E2 enzymes and subjected to immunoblotting with anti-HA antibody to identify the auto-ubiquitylation. (B) DDB2 was directly ubiquitylated by the DDB–Cul4A complex. Pure DDB1–DDB2-Cul4A complex was subjected to in vitro ubiquitylation assay in the absence (no) or presence of ubiquitin (Ub) or GST-ubiquitin (GST-Ub) and analyzed by immunoblotting with each antibody. Single asterisks show the ubiquitin conjugation and double asterisks indicate GST-ubiquitin conjugation.

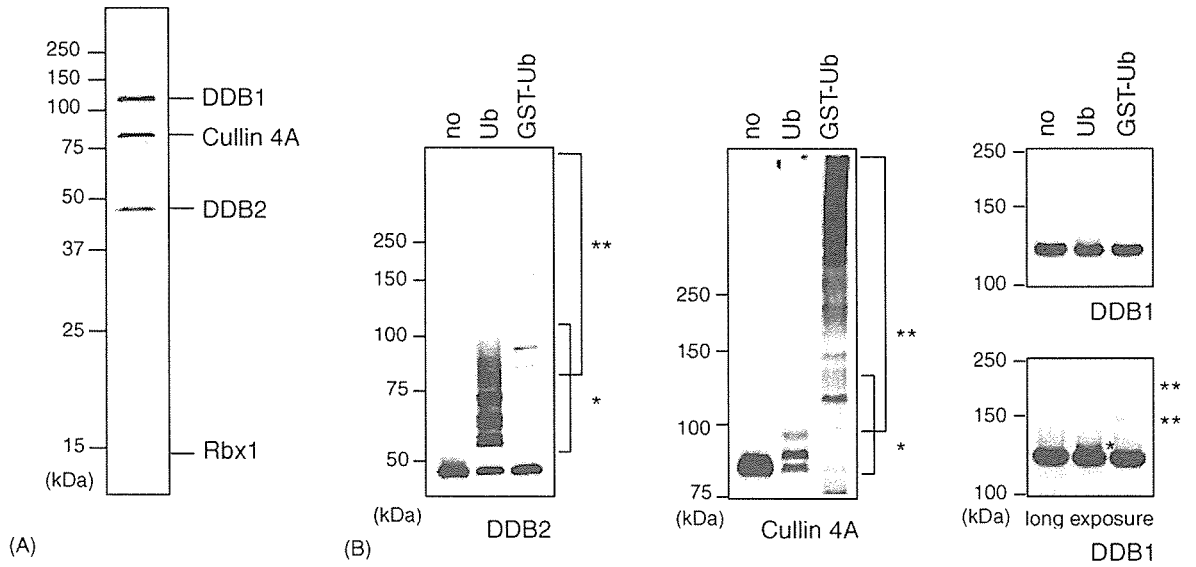

Fig. 4. In vitro ubiquitylation of DDB2 using authentic DDB–Cul4A complex. (A) Purification of the genuine DDB–Cul4A complex. Purified complex was resolved by SDS-PAGE and visualized by silver staining. (B) In vitro ubiquitylation of DDB2 and auto-ubiquitylation of Cul4A. The authentic DDB–Cul4A complex was subjected to in vitro ubiquitylation assay in the absence (no) or presence of ubiquitin (Ub) or GST-ubiquitin (GST-Ub) and analyzed by immunoblotting with anti-HA (DDB2), anti-Cul4A and anti-DDB1 antibodies. Single asterisks show the ubiquitin conjugation and double asterisks indicate GST-ubiquitin conjugation.

lation of DDB2 and Cul4A were again observed (Fig. 4B, single asterisk). The exclusion of ubiquitin from the assay quenched these bands and replacement of native ubiquitin with GST-ubiquitin retarded their mobility (Fig. 4B, double asterisks). In contrast, DDB1 was rarely ubiquitylated, although a faint ubiquitylation signal was observed after long exposure (Fig. 4B, right panel). Because the DDB–Cul4A complex derived from both insect (Fig. 3) and mammalian (Fig. 4) cells directly ubiquitylated DDB2, we concluded that DDB2 was ubiquitylated by genuine DDB–Cul4A complex.

3.4. XP-E mutation does not affect ubiquitylation of DDB2 in vitro

Two cell lines established from XP-E patients, XP2RO and XP82TO, have been characterized in detail. XP2RO and XP82TO cells harbor naturally occurring single amino acid substitutions, R273H and K244E, in DDB2 protein, respectively. It has been reported that the XP82TO mutant protein (DDB2-K244E) interacts normally with DDB1 and Cullin 4A. Conversely, XP2RO mutant protein (DDB2-R273H) interacts with neither of them [13,29]. We also confirmed by immunoprecipitation experiments that DDB2-K244E interacts with DDB1 and Cullin 4A normally, but DDB2-R273H did not associate with either of them (Fig. 5A). Intriguingly, Rapic-Otrin et al. [21] reported that UV-induced rapid degradation of DDB2 protein did not occur in XP82TO cell line. This information prompted us to test whether K244E mutation affects the in vitro ubiquitylation of DDB2. Because DDB1–DDB2 (K244E)-Cul4A complex did not interact effectively with DNA cellulose, we were unable to purify it compared with the wild-type complex (data not shown). We thus purified this mutant protein complex by affinity chromatography on nickel-chelating column and subsequent heparin column, and the bound DDB2 (K244E) complex was eluted around 0.5 M NaCl (Fig. 5B). As a control, the wild type DDB2-containing complex was simultaneously isolated by the same method. The DDB1–DDB2 (K244E)-Cul4A complex was incubated with ATP, ubiquitin, E1 and UbcH5a, and subjected to immunoblotting with the anti-Flag antibody. The mutant DDB2 protein was ubiquitylated in a manner equivalent to that of the wild-type control (Fig. 5C), indicating that XP82TO mutation (K244E) did not affect the ubiquitylation of DDB2 in vitro. This result also suggests that the mutated site of DDB2 (244th K) per se is not the unique ubiquitylation site.

4. Discussion

The DDB complex is regulated through several processes when cells are exposed to UV irradiation, namely very rapid translocation into the nucleus and binding to chromatin [17,29–31], hasty degradation of DDB2 protein [21,22] and final transcriptional induction of *DDB2* mRNA [32,33]. Chemical inhibition of proteasomes prevents rapid degradation of DDB2 protein, suggesting that this process is mediated by the ubiquitin/proteasome system. Among these regulation processes of DDB2, proteolytic degradation is the most intriguing because several recent reports [13,15–17] and our present results have shown a tight relationship between the DDB complex and proteins involved in ubiquitylation.

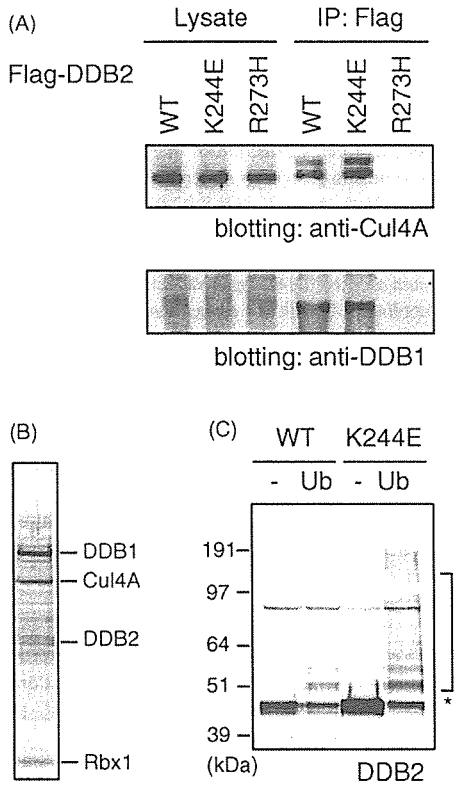

Fig. 5. XP82TO mutation (K244E) does not affect the in vitro ubiquitylation of DDB2. (A) DDB2-K244E interacts with DDB1 and Cul4A, but DDB2-R273H associates with neither. Flag-DDB2 (WT, K244E or R273H) was co-transfected with Myc-Cul4A and HA-DDB1 into ts41 cells. After immunoprecipitation (IP) by anti-Flag antibody, the resulting immunoprecipitates were subjected to immunoblotting using anti-Cul4A and anti-DDB1 antibodies. (B) The DDB2 (K244E) complex was resolved by SDS-PAGE and visualized by silver staining. (C) Ubiquitylation of DDB2 protein with XP-E mutation (DDB2-K244E) was comparable with that of wild-type DDB2 in vitro. The DDB–Cul4A complex containing mutant or wild type DDB2 was subjected to in vitro ubiquitylation in the presence (Ub) or absence (-) of ubiquitin. Asterisk indicates the ubiquitin-conjugated DDB2.

Interestingly, ectopic over-production of Cullin 4A accelerates the degradation of DDB2, suggesting that Cul4A ubiquitylates DDB2 [15,16]. However, no reconstitution experiments were performed and thus this information did not exclude the possible involvement of other E3(s) downstream of Cul4A in the ubiquitylation of DDB2, rather than directly by Cul4A. This situation prompted us to reconstitute the in vitro ubiquitylation of DDB2 and we presented in this study biochemical evidence for the ubiquitylation of DDB2 directly by the DDB–Cullin 4A complex.

4.1. In vitro ubiquitylation of each subunit of the DDB complex

It is well established that a significant fraction of DDB2 is degraded promptly after UV irradiation [21,22] and is also degraded in a cell cycle-dependent manner [16]. Conversely, it is still controversial whether another component of the DDB complex, DDB1, is a target of ubiquitylation and subsequent degradation. Zhou's group reported that overproduction of Cul4A in cells stimulates the ubiquitylation of DDB1 [15]. In contrast, neither ectopically expressed Cul4A nor UV irradiation accelerates degradation of DDB1 was reported by other groups [12,16]. In our reconstitution experiment, DDB1 was very weakly ubiquitylated in the DDB–Cul4A complex from insect cells (Fig. 3B) and seldom ubiquitylated in the complex from HeLa cells (Fig. 4B). Because the HeLa cell-derived complex is purer and was considered to be isolated under more physiological conditions, this result supports the notion that DDB1 is not ubiquitylated by the Cul4A E3 complex. Even though DDB1 was faintly ubiquitylated, such mono- or di-ubiquitylation is insufficient for the proteasomal degradation. Therefore, we favor the scenario that not DDB1 but DDB2 is the target of ubiquitylation by Cul4A E3 complex in vivo [12,16].

4.2. XP-E mutation did not affect the ubiquitylation of p48 in vitro

Rapic-Otrin et al. [21] reported that UV-induced rapid degradation of DDB2 did not occur in the XP-E cell line (XP82TO) whose DDB2 harbors a K244E mutation. Because this mutant protein (DDB2-K244E) can interact with DDB1 and Cullin 4A (Fig. 5A [13]) but not with damaged DNA [8,12,34], this result suggests that the binding activity to damaged-DNA is necessary for the degradation of DDB2. Another possibility is that the mutated site of DDB2 (244th K) per se is the main ubiquitylation site, as suggested previously [21]. However, the latter is unlikely because we showed that this mutant protein was still ubiquitylated in a manner similar to the wild-type DDB2 protein in vitro (Fig. 5C). Perhaps binding to damaged-DNA renders the conformation of DDB complex more acquiescent for ubiquitylation and/or UV recruits DDB to some specialized chromatin place where the other ubiquitylation machinery is easy to access in vivo.

4.3. Biochemical role of DDB in nucleotide excision repair

In XP-E cells lacking the DDB activity, the nucleotide excision repair (NER) of cyclobutane pyrimidine dimer (CPD) is significantly impaired [32], suggesting the importance of DDB complex in NER in vivo. However, this DDB complex is not essential for the reconstitution of the cell-free NER in vitro. The NER reaction was successfully reconstituted in the absence of DDB [35–37], although it may exhibit some stimulatory or inhibitory effects under certain conditions [38–40]. One interpretation of these results is that some partner protein(s) of DDB complex may be missing in such NER assay in vitro. Recent studies [13,15–20,41–43] and the present work emphasize the role of the DDB1 complex in the ubiquitin ligation. We can thus speculate that the effect and requirement of DDB could change if other ubiquitylation machinery was added to the in vitro NER assay.

4.4. Biological function of DDB2 ubiquitylation

As mentioned above, DDB2 protein is rapidly degraded after UV-irradiation in vivo [21,22] and we showed here that the DDB–Cul4A complex could directly ubiquitylate DDB2. What is the function of DDB2 ubiquitylation and subsequent degradation? After damaged-DNA recognition, DDB is thought to hand over the DNA lesion to the following NER component(s) including XPC [2]. An appealing hypothesis is that clearance of DDB2 by ubiquitylation and succeeding degradation facilitates accession of the following NER factor(s) to the DNA lesion. However, it is not clear at present whether the ubiquitylation of DDB2 is only required for its UV-induced degradation, or is essential to change some biological character of DDB2 preceding degradation. Because various non-proteolytic functions of ubiquitylation have been identified recently [44], it is still conceivable that the ubiquitylation of DDB2 might have an additional role besides degradation. Moreover, we still do not know whether DDB2 ubiquitylation is a pertinent event for DDB function in DNA repair, or is the only side effect accompanied by ubiquitylation of authentic, relevant substrate. To define the precise role of DDB–Cullin 4A complex-mediated ubiquitylation, further studies are obviously required; especially the identification of the physiological substrate. Such experiments are currently underway in our laboratories.

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Glycoprotein-specific ubiquitin ligases recognize *N*-glycans in unfolded substrates

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Misfolded or unassembled polypeptides in the endoplasmic reticulum (ER) are retro-translocated into the cytosol and degraded by the ubiquitin–proteasome system. We reported previously that the $SCF^{Fbs1,2}$ ubiquitin-ligase complexes that contribute to ubiquitination of glycoproteins are involved in the ER-associated degradation pathway. Here we investigated how the $SCF^{Fbs1,2}$ complexes interact with unfolded glycoproteins. The SCF^{Fbs1} complex was associated with p97/VCP AAA ATPase and bound to integrin-β1, one of the SCF^{Fbs1} substrates, in the cytosol in a manner dependent on p97 ATPase activity. Both Fbs1 and Fbs2 proteins interacted with denatured glycoproteins, which were modified with not only high-mannose but also complex-type oligosaccharides, more efficiently than native proteins. Given that Fbs proteins interact with innermost chitobiose in *N*-glycans, we propose that Fbs proteins distinguish native from unfolded glycoproteins by sensing the exposed chitobiose structure.

Keywords: ubiquitin ligase; glycoprotein; unfold; *N*-glycan

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INTRODUCTION

Most secretory and membrane proteins are co-translationally translocated into the lumen of the endoplasmic reticulum (ER). In the ER, these proteins are modified by *N*-linked oligosaccharides and subjected to 'quality control' in which aberrant proteins are distinguished from properly folded proteins (Ellgaard & Helenius, 2003). When the improperly folded or incompletely assembled proteins fail to restore their functional states, they are degraded by the ER-associated degradation (ERAD) system, which involves retrograde transfer of proteins from the ER to the cytosol followed by degradation by the proteasome. p97/VCP, a cytosolic ATPase member of the AAA ATPase family, may have several roles in the ERAD pathway. It has been suggested that the p97–Ufd1–Npl4 complex is required for the extraction of misfolded proteins from the ER into the cytosol (Tsai *et al*, 2002). Such a retro-translocation step would most probably be mediated by dual recognition of the substrates by p97, with the complex binding both the nonubiquitinated segment of a substrate and the attached polyubiquitin chain (Ye *et al*, 2003). The p97 complex, which associates with the ER membrane proteins VIMP and Derlin-1 through the amino-terminal domain of p97, is thought to be required for the extraction of misfolded proteins from the ER (Lilley & Ploegh, 2004; Ye *et al*, 2004). However, p97 has also been proposed to be important for the release of ERAD substrates after their export from the ER, and thereby for their accessibility to the proteasome (Elkabetz *et al*, 2004). The role of each of these functions remains to be established.

In the ubiquitin system, the ubiquitin ligase 'E3' has an important role in the selection of target proteins for ubiquitination. At present, several E3s have been identified in the ERAD pathway, such as Hrd1 (Bays *et al*, 2001) and Doa10 (Swanson *et al*, 2001) in yeast, and gp78 (Fang *et al*, 2001), CHIP (Meacham *et al*, 2001) and Parkin (Imai *et al*, 2001) in mammals. In addition, we have recently identified a new member of the ERAD-linked E3 family, SCF^{Fbs}, which participates in ERAD for selective elimination of glycoproteins (Yoshida *et al*, 2002, 2003). Whereas Hrd1, Doa10 and gp78 are localized in the ER, SCF^{Fbs} complexes are localized in the cytosol similar to CHIP and Parkin.

The SCF is composed of Cullin1/Cdc53, Skp1, Roc1/Rbx1, and one member of the large family of F-box proteins, which are involved in trapping target proteins (Deshaies, 1999). Fbs1 and Fbs2 (F-box protein that recognizes sugar chains) interact with glycoproteins containing high-mannose oligosaccharides, protein modification of which occurs in the ER. Our recent X-ray crystallographic and nuclear magnetic resonance (NMR) studies of the substrate-binding domain of Fbs1 have shown that Fbs1 recognizes the inner chitobiose of high-mannose oligosaccharides by a small hydrophobic pocket located at the top of the β-barrel (Mizushima *et al*, 2004).

In this study, we examined whether Fbs proteins discriminate between folded and unfolded glycoproteins because ERAD substrates are thought to be unfolded. Both Fbs1 and Fbs2 preferably bind to denatured proteins that contain not only

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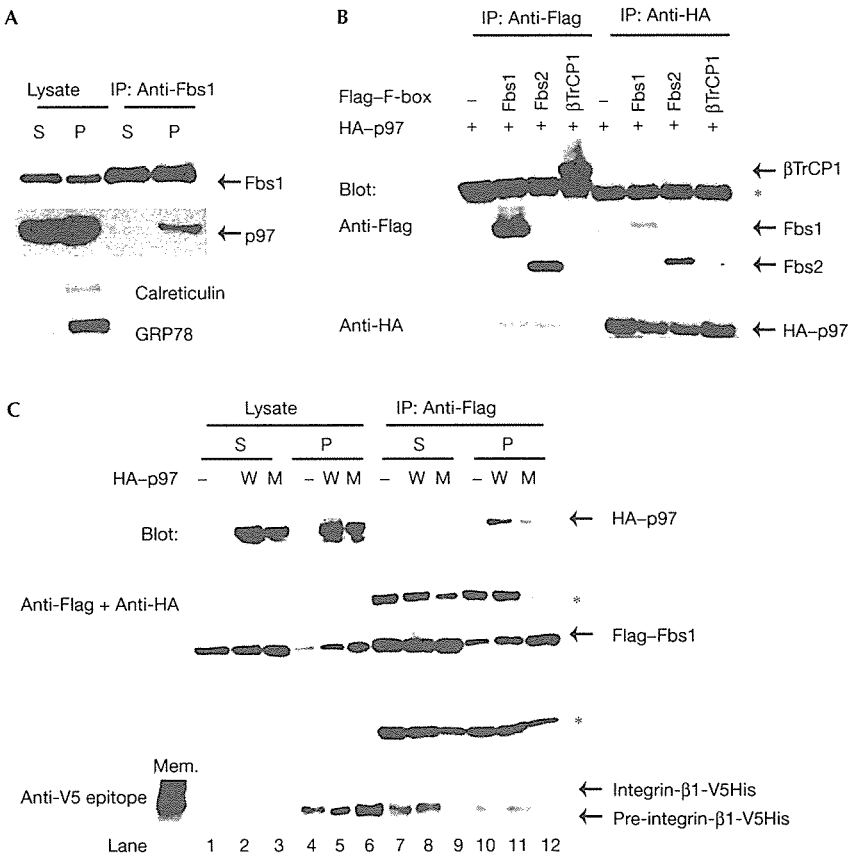

Fig 1 | Fbs1 binds to integrin-β1 dependent on p97 ATPase activity. (**A**) Fbs1 associates with p97 in microsomal fraction. Endogenous Fbs1 was immunoprecipitated from 100,000*g* supernatant (S) and precipitate (P) fractions of brains of adult mice. Lysate (15 μg each) and immunoprecipitates were analysed by immunoblotting with antibodies against Fbs1, p97, calreticulin and GRP78. (**B**) Interaction of Fbs proteins with p97. Lysates of 293T cells transiently expressing Flag-tagged F-box proteins (–, empty vector) and HA-tagged p97 were subjected to immunoprecipitation, and the resulting precipitates were analysed by immunoblotting. The asterisk shows immunoglobulin heavy chains. (**C**) Fbs1 binding to integrin-β1 in the cytosol depends on p97 ATPase activity. 293T cells were transfected with Flag-tagged Fbs1, V5-tagged integrin-β1 and HA-tagged p97 (–, empty vector; W, wild-type p97; M, mutant p97 (K524A)). Fbs1 was immunoprecipitated from supernatant (S) and precipitate (P) fractions. Expressions of p97, Fbs1 and integrin-β1 in fractionated lysates (5 μg each) and the amount of integrin-β1 associated with Fbs1 were analysed by immunoblotting using anti-V5 antibody. The membranous fraction (Mem.) was prepared from 24,000*g* precipitate. Asterisks show immunoglobulin heavy and light chains.

high-mannose but also complex-type oligosaccharides over native counterparts. The results showed that these F-box proteins probably interact with the innermost chitobiose in *N*-glycans in only unfolded glycoprotein in the ERAD pathway, considering that chitobiose moieties are usually masked by the folded polypeptide.

RESULTS

SCF^{Fbs} associates with the p97 complex

We have isolated Fbs1 from mouse brain cytosol as a novel sugar-binding protein that functions as a substrate-binding subunit in SCF-type E3 for ERAD (Yoshida *et al*, 2002), but the ubiquitination machinery for ERAD is probably associated with the ER membrane. To test the localization of Fbs1 proteins in mouse brain, we prepared anti-Fbs1 polyclonal antibodies. Lysates from adult mouse brain were fractionated into 100,000*g* supernatant (S) and precipitate (P) fractions excluding 24,000*g* precipitate, and the presence of Fbs1 was analysed by immunoblotting (Fig 1A). Fbs1 was detected in the P as well as the S fractions, suggesting that Fbs1 interacts with proteins that associate with the ER membrane. As p97/VCP is thought to be involved in the retro-transport of ERAD substrates (Tsai *et al*, 2002), we examined the interaction of Fbs1 with p97. As shown in Fig 1A, Fbs1 was co-immunoprecipitated with p97 from the P but not from the S fraction. To determine whether other F-box proteins interact with p97, the Flag-tagged F-box proteins were expressed, together with haemagglutinin (HA)-tagged p97, in 293T cells, immunoprecipitated and analysed by immunoblotting (Fig 1B). Both Fbs1 and Fbs2 but not βTrCP1, an F-box protein with WD repeats for substrate recognition, were co-immunoprecipitated with p97. These results suggest that a part of Fbs proteins binds specifically to the p97-containing complex at the ER.

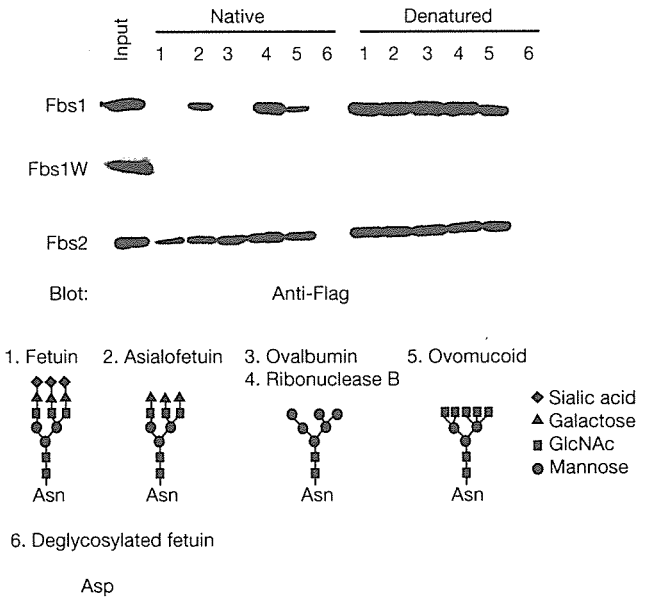

Fig 2 | Pull-down analysis of the interactions of Fbs1, Fbs1W and Fbs2 with native and denatured *N*-glycoproteins. Extracts of cells expressing Flag-tagged Fbs1, the W280A mutant of Fbs1 (Fbs1W) and Fbs2 were incubated with native or guanidine-HCl-treated (denatured) glycoproteins (lanes 1–5) or deglycosylated fetuin (lane 6)-immobilized beads. The beads were washed and then boiled with sample buffer. Lysates (7.5 μg) and bound proteins were analysed by immunoblotting using anti-Flag antibody (top) and the structures of *N*-glycans in the glycoproteins tested are shown at the bottom.

Fbs1 binds to integrin-β1 dependent on p97 activity

We identified pre-integrin-β1, which was modified with high-mannose oligosaccharides, as one of the Fbs1 substrates (Yoshida *et al*, 2002). As the Fbs1–pre-integrin-β1 interaction occurs in the cytosol, retro-translocation of integrin-β1 from the ER into the cytosol is required for Fbs1 binding. To analyse the involvement of p97 in the interaction between Fbs1 and pre-integrin-β1, 293T cells were transfected with expression plasmids encoding Flag-tagged Fbs1, V5-tagged integrin-β1 together with HA-tagged p97 or its mutant, and treated with the proteasome inhibitor MG132 for 1.5 h before collecting cells. Immunoprecipitates with anti-Flag antibody of S and P fractions of transfected 293T cells were immunoblotted with anti-V5 antibody to detect co-immunoprecipitated integrin-β1 (Fig 1C). Most of pre-integrin-β1 was localized in the P fractions, and the expression of the ATPase-defective p97 mutant (K524A) increased the amount of total pre-integrin-β1 in the P fraction (lanes 4–6). The amount of pre-integrin-β1 associated with Fbs1 was greater in wild-type p97-expressing cells than in mutant p97-expressing cells in both S and P fractions (lanes 7–12). These results suggest that Fbs proteins recognize and ubiquitinate pre-integrin-β1 retro-translocated by p97, and this modification may facilitate pre-intergrin-β1 binding to the p97–Ufd1–Npl4 complex, as well as its extraction from the ER.

Fbs interacts with denatured *N*-glycoproteins

Glycoproteins retro-translocated from the ER are not native proteins. Therefore, to examine whether Fbs proteins recognize denatured glycoproteins better than native proteins, first we carried out a pull-down assay using several *N*-glycoproteins (Fig 2). We have reported that both Fbs1 and Fbs2 recognize the innermost chitobiose structure in high-mannose oligosaccharides (Yoshida *et al*, 2003). Although both ovalbumin and ribonuclease B (RNaseB) contain high-mannose oligosaccharides, Fbs1 effectively bound to RNaseB alone (see Discussion). Fbs1 could bind to asialofetuin and ovomucoid, but could hardly bind to fetuin and ovalbumin. The ability of Fbs2 to bind to asialofetuin, fetuin and ovomucoid was weaker than that to proteins attached to the high-mannose oligosaccharides. Interestingly, both Fbs proteins could bind to all the denatured *N*-glycoproteins tested but not to denatured deglycosylated proteins (lane 6), whereas the W280A mutant of Fbs1 (Fbs1W) that fails to interact with the innermost GlcNAc moiety in *N*-glycan (Mizushima *et al*, 2004) could not bind to any native or denatured glycoproteins, suggesting that the denaturation of glycoproteins increases the accessibility to the innermost chitobiose of *N*-glycans by Fbs proteins.

We next examined whether the binding potency and substrate specificity of Fbs proteins are influenced by denaturation of cellular glycoproteins. Lysates from the mouse brain or Neuro2a cells were treated with or without 6 M guanidine-HCl, diluted ten times with the lysis buffer and incubated with the His-tagged Fbs proteins produced by *Escherichia coli* (Fig 3; supplementary information 1 online). Guanidine-HCl at 0.6 M had no influence on Fbs binding to glycoproteins (supplementary information 2 online). The glycoproteins bound to Fbs were isolated using Ni-NTA affinity chromatography and detected by lectin blotting (Fig 3). Blotting with GNA, a lectin that binds to high-mannose oligosaccharide, showed that denaturation markedly increased the number of proteins bound to Fbs. The spectrum of Fbs1-bound protein bands in the brain detected by WGA, a lectin specific for terminal GlcNAc or sialic acids, was similar to those detected by GNA, suggesting that these proteins are modified by both high-mannose and complex-type oligosaccharides. Conversely, the proteins detected by RCA120, a lectin that binds to terminal galactose-β1-4GlcNAc, were different to those detected by GNA. Both the quantities and species of RCA120-reactive proteins recognized by Fbs1 were also considerably increased by denaturation. Treatment of denatured proteins with peptide: *N*-glycanase (PNGase F) almost diminished their binding to Fbs. Furthermore, Fbs1W could hardly bind these glycoproteins. These results strongly suggest that both Fbs1 and Fbs2 bind to the innermost GlcNAc moiety irrespective of the terminal sugar moieties, and that the accessibility of Fbs proteins to the innermost GlcNAc moiety is enhanced by denaturation of the substrate glycoproteins. As all *N*-linked oligosaccharides contain innermost chitobiose structure, Fbs proteins seem to be capable of binding most *N*-glycoproteins when denatured.

SCF^{Fbs1} ubiquitinates denatured glycoproteins

To see whether SCF^{Fbs} ubiquitinates denatured glycoproteins more efficiently than native counterparts, we performed an *in vitro* ubiquitination assay using purified components including recombinant SCF^{Fbs1} proteins. Efficient ubiquitination of GlcNAc-terminated fetuin (GTF), which is an *in vitro* substrate for SCF^{Fbs1} (Yoshida *et al*, 2002), was detected by immunoblotting using an anti-fetuin antibody. When an excess amount of substrates existed, denatured asialofetuin was efficiently ubiquitinated,

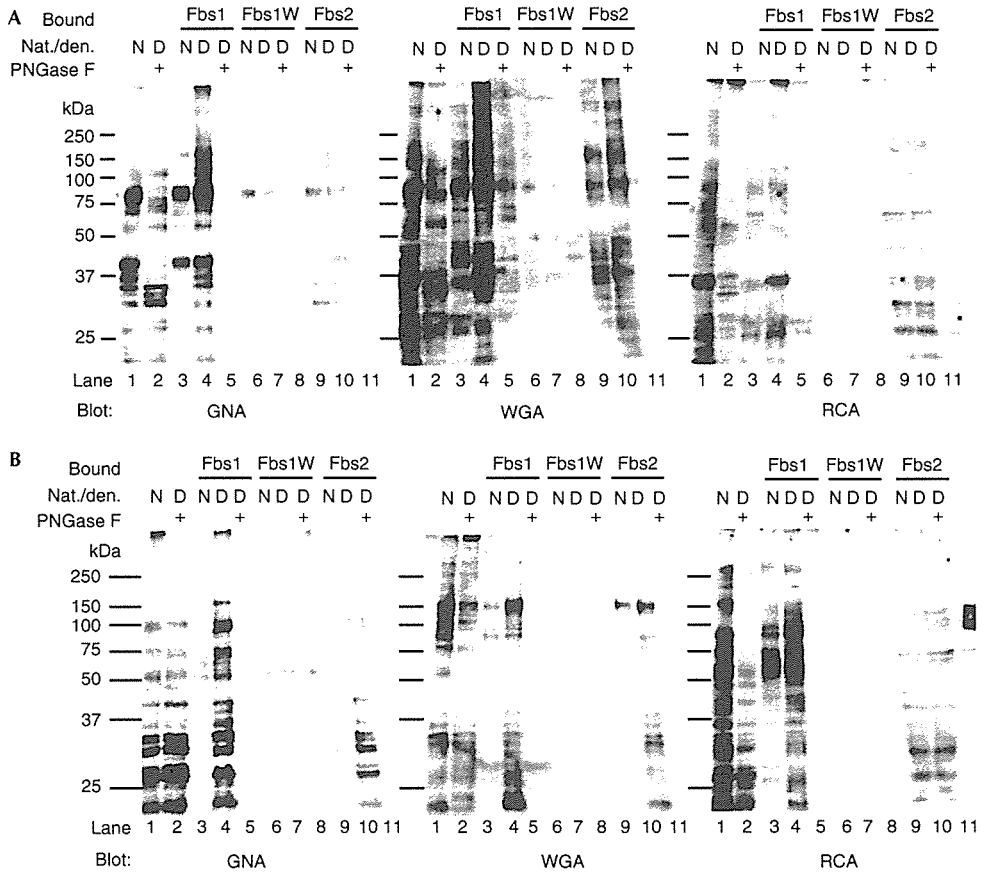

Fig 3 | Interactions of Fbs1 and Fbs2 with cellular glycoproteins containing *N*-linked oligosaccharides in native and denatured states. Native (N), denatured (D) or PNGase-treated proteins prepared form mouse brain (**A**) or Neuro2a cells (**B**) were incubated with recombinant Fbs1-, Fbs1W- and Fbs2-immobilized beads. Native and PNGase-treated lysates (15 μg each; lanes 1 and 2) and proteins bound to Fbs1, Fbs1W or Fbs2 were analysed by lectin blotting using HRP-labelled GNA, WGA and RCA.

whereas ubiquitination of native asialofetuin was marginal (Fig 4; supplementary information 3 online). No ubiquitination of GTF or denatured asialofetuin was detected in the absence of E1, E2, ATP or substrate, and SCF^{Fbs1W} (Mizushima *et al*, 2004) failed to ubiquitinate these substrates (supplementary information 4 online). These results demonstrate that the higher affinity of Fbs1 for denatured *N*-glycoproteins results in a more efficient ubiquitination of the denatured substrates than native counterparts.

DISCUSSION

In the early secretory pathway, *N*-glycosylation facilitates conformational maturation by promoting the glycoprotein-folding machinery, and functions as tags for ER retention and targeting to the ERAD pathway. The calnexin–calreticulin cycle, consisting of two homologous lectins, calnexin and calreticulin, which interact with monoglucosylated *N*-glycans, in concert with UDP-glucose:glycoprotein glucosyltransferase (GT) and glucosidase II, has a central role in folding and ER retention. Conversely, it is shown that α-mannosidase I and EDEM have a pivotal role in selective disposal of misfolded glycoproteins (Ellgaard & Helenius, 2003; Yoshida, 2003). Among these oligosaccharide-related molecules in the ER, only GT has been shown to recognize incompletely folded proteins (Parodi, 2000). In the cytosol, the *N*-glycans in proteins extracted from the ER are removed before proteolysis by PNGase. In addition, PNGase can discriminate between non-native and folded glycoproteins, favouring the former (Hirsch *et al*, 2004). In this study, we showed that the Fbs proteins preferentially bind to denatured glycoproteins over properly folded proteins. As the retro-translocated proteins in the cytosol from the ER are misfolded, it is conceivable that *N*-glycan recognition proteins in the cytosol can sense misfolded states.

Although GT and PNGase can distinguish the folding states of substrates, the structural elements required for identification of their targets are not fully understood. *In vitro* studies have shown that GT also preferentially re-glucosylates glycoproteins in partially folded, molten globule conformations (Caramelo *et al*, 2003), and that an important feature for recognition is the exposure of hydrophobic clusters and innermost GlcNAc residue (Sousa & Parodi, 1995). Fbs1 interacts with the inner chitobiose in *N*-glycans of glycoproteins by a specific binding surface located at one tip of the β-sandwich of its substrate-binding domain (Mizushima *et al*, 2004). The intramolecular interactions of

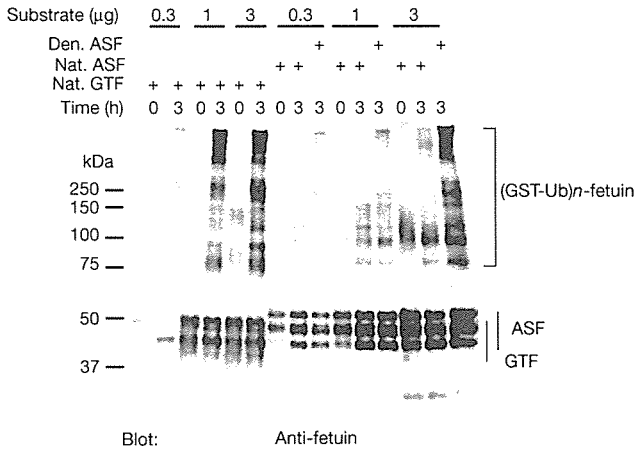

Fig 4 | *In vitro* ubiquitination of native GlcNAc-terminated fetuin (GTF), asialofetuin (ASF) and denatured ASF by SCF^{Fbs1} ligase. The high-molecular-mass ubiquitinated fetuin ((GST-Ub)*n*-fetuin) was detected by immunoblotting with anti-fetuin antibody.

innermost GlcNAc residue and the polypeptide moiety generally hamper the binding of Fbs1 to the chitobiose portions of glycoproteins as a result of steric hindrance in their native states. Therefore, Fbs1 recognizes the innermost position of *N*-glycans as a signal for unfolded glycoproteins. Conversely, as RNaseB contains an oligosaccharide that does not contact the polypeptide chain except at the covalent attachment point (Williams *et al*, 1987), it is likely that RNaseB interacts with Fbs1 even in the native form probably due to the exceptional freedom of the innermost chitobiose portion (Fig 2). The present results confirmed that Fbs proteins bind to denatured glycoproteins.

Considering that ubiquitination of ERAD substrates is linked to retro-translocation and rapid degradation, ubiquitin ligases for ERAD might be associated with the ER membrane. Moreover, as many glycoproteins are efficiently deglycosylated by PNGase after retrograde transfer into the cytosol (Blom *et al*, 2004), the rapid recognition of substrate by Fbs proteins before deglycosylation is critical. We found a part of Fbs proteins in association with p97 in the microsomal fractions, and extraction of the substrate of SCF^{Fbs1} was dependent on the ATPase activity of p97 followed by association with Fbs1. Thus, SCF^{Fbs} seems to be positioned in the ER membrane in such a way that it can ubiquitinate substrates immediately after retro-translocation to the cytosol.

Fbs proteins can bind not only high-mannose oligosaccharides but also various types of *N*-glycans in glycoproteins. These modified glycoproteins other than the high-mannose oligosaccharides are not ERAD substrates. Therefore, our finding suggests that SCF^{Fbs} mediates ubiquitination of exogenous or membrane proteins endocytosed into the cells. This is not unusual, because it is well known that extracellular proteins incorporated by phagocytosis into dendritic cells are presented to MHC class I molecules after proteasomal degradation (Castellino *et al*, 2000). Other studies demonstrated the transfer of endocytosed proteins into the cytosol by unknown mechanisms before their proteasomal processing and/or destruction (Kovacsovics-Bankowski & Rock, 1995). Further functional analysis of Fbs family proteins can shed light on the degradation of endocytosed proteins.

METHODS

Transfection, plasmids, antibodies, immunoprecipitation and immunoblotting. 293T cells were transfected as described previously (Yoshida *et al*, 2002). HA–p97-expressing plasmid was a kind gift from S. Khochbin (INSERM, France). HA–p97 (K524A)-expressing plasmid was constructed by site-directed mutagenesis. Human integrin-β1 complementary DNA was cloned from cDNA clone (ATCC 988953) in pTracer-EF-V5His vector (Invitrogen, Carlsbad, CA, USA). The anti-mouse Fbs1 serum was generated in rabbits by standard procedures using a synthetic peptide corresponding to residues 1–14 (MDGDGDPFSVSHPE) of the predicted protein coupled to keyhole limpet haemocyanin. Monoclonal antibodies to p97 and V5 epitope were purchased from Progen (Heidelberg, Germany) and Invitrogen, respectively, and polyclonal antibodies to calreticulin and GRP78 were from Affinity Bioreagents (Exeter, Devon, UK). Antibodies to Flag, HA and fetuin have been described previously (Yoshida *et al*, 2003). Immunoprecipitation from whole-cell extracts or subcellular fractionation of cells and immunoblotting were performed in TBS-T (1% Triton X-100, 50 mM Tris–HCl pH 7.5, 150 mM NaCl and protease inhibitors) as described previously (Yoshida *et al*, 2002).

Pull-down assay. Fetuin, asialofetuin, ovalbumin, RNaseB and ovomucoid were purchased from Sigma-Aldrich (St Louis, MO, USA). For preparation of deglycosylated fetuin (DGF), 10 mg of asialofetuin was incubated with 200 U of PNGase F (Roche, Mannheim, Germany) in 50 mM phosphate buffer, pH 7.2, at 37 °C for 24 h. The enzyme-treated proteins were loaded onto successive WGA and RCA-lectin agarose columns. The flow-through fraction from both columns was used as DGF. Each 10 mg glycoprotein was immobilized to 0.5 ml of Affi-gel 10 or 15 (Bio-Rad, Richmond, CA, USA). For preparation of denatured-glycoprotein-immobilized beads, after each half of glycoprotein-immobilized beads was incubated in 6 M guanidine-HCl for 2 h, the beads were washed five times with ten volumes of 20 mM Tris–HCl (pH 7.5)/150 mM NaCl (TBS) containing 0.5% NP-40 (TBS-N). Each cell extract prepared with TBS-N from Flag-tagged Fbs1, Fbs1 W280A mutant or Fbs2-expressing 293T cells (30 μg) was incubated with 15 μl of various glycoprotein-immobilized beads. Bound proteins were eluted by boiling with SDS sample buffer and were analysed by immunoblotting.

Binding assay and lectin blotting. The substrate-binding domain of mouse Fbs1 (117–297) and its W280A mutant were cloned into pET15b (Mizushima *et al*, 2004), that of mouse Fbs2 (46–295) was cloned into pET33b, and expressed in *E. coli*. The His-tagged Fbs proteins were bound to Ni-NTA agarose beads (Qiagen, Hilden, Germany). Mouse brains and Neuro2a cells were homogenized in TBS-N and protease inhibitors. After centrifugation of the homogenate at 15,000*g* for 30 min, guanidine-HCl was dissolved with one-third of the supernatant (protein concentration 5 mg ml^{-1}) up to 6 M. Guanidine-HCl-treated and untreated lysates were diluted ten times with TBS-N. Another aliquot was treated with PNGase F subsequent to denaturation by heating for 5 min at 100 °C in the presence of 1% SDS and was then diluted ten times with TBS-N. The dilutes and PNGase-treated lysates were precleared with Ni-NTA agarose and then the flow-through fractions were incubated with the Fbs-protein-bound beads for 18 h at 4 °C. The beads were washed with TBS-N containing 20 mM imidazole. The adsorbed proteins were eluted by 0.2 M

imidazole in TBS-N. Eluted proteins were separated by SDS–PAGE, and blotted onto a membrane (Immobilon). After the blotted membranes were blocked with 3% bovine serum albumin in PBS, lectin blotting was performed using horseradish peroxidase (HRP)-labelled GNA (EY Laboratories), RCA120 and WGA (Seikagaku-kogyo, Japan).

***In vitro* ubiquitination assays.** Preparation of GTF and *in vitro* ubiquitination assays were performed as described previously (Yoshida *et al*, 2002). Denatured asialofetuin was prepared by 200 times dilution of 20 mg ml^{-1} asialofetuin treated with 6 M guanidine-HCl. Details of the assay condition are described in supplementary information 5 online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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Co-chaperone CHIP Associates with Expanded Polyglutamine Protein and Promotes Their Degradation by Proteasomes*

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A major hallmark of the polyglutamine diseases is the formation of neuronal intranuclear inclusions of the disease proteins that are ubiquitinated and often associated with various chaperones and proteasome components. But, how the polyglutamine proteins are ubiquitinated and degraded by the proteasomes are not known. Here, we demonstrate that CHIP (C terminus of Hsp70-interacting protein) co-immunoprecipitates with the polyglutamine-expanded huntingtin or ataxin-3 and associates with their aggregates. Transient overexpression of CHIP increases the ubiquitination and the rate of degradation of polyglutamine-expanded huntingtin or ataxin-3. Finally, we show that overexpression of CHIP suppresses the aggregation and cell death mediated by expanded polyglutamine proteins and the suppressive effect is more prominent when CHIP is overexpressed along with Hsc70.

The pathological expansion of unstable trinucleotide repeats has been found to cause 15 neurological diseases, 9 of which are neurodegenerative diseases (also referred to as polyglutamine diseases) resulting from the expansion of CAG repeats within the coding region of the responsible genes. Those nine include Huntington's disease (HD),[1] dentatorubral pallidoluysian atrophy, X-linked spinal bulbar muscular atrophy (SBMA), and several spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17). All nine disorders are progressive, dominantly inherited (except spinal bulbar muscular atrophy), typically begin in midlife, and result in severe neuronal dysfunction and neuronal cell death. Increasing length of glutamine repeats in the affected individual strongly correlates with earlier age of onset and disease severity (1).

Evidence suggests a toxic gain-of-function effect of the polyglutamine expansion on the protein, and this novel neurotoxic property most likely involves an increased propensity for the disease protein to aggregate (2). In human disease tissue, transgenic animal models, and transfected cells expanded polyglutamine proteins have been shown to undergo intracellular aggregation, in most cases forming neuronal intranuclear inclusions (3). However, the discovery of ubiquitinated aggregates or the neuronal intranuclear inclusions and the association of various chaperones and proteasome components with the aggregates suggest that the cells recognize the aggregated disease protein as abnormal and may represent an appropriate cellular response to refold or degrade aggregated mutant protein (4–9). Consistent with this idea, it has been experimentally demonstrated that overexpression of selective chaperones in the mammalian cell culture suppresses the aggregate formation and cell death (4, 6, 7, 9) and that the proteasome system is indeed involved in the degradation of polyglutamine proteins (5, 10, 11). However, very little is known about the delivery of the expanded-polyglutamine proteins to the ubiquitin proteasome pathway (UPP) for degradation.

In the present investigation, we studied the detail mechanism of ubiquitination of the expanded polyglutamine proteins using polyglutamine-expanded truncated N-terminal huntingtin (tNhtt) as well as truncated ataxin-3 as models. We found that CHIP, an ubiquitin ligase, associates with the expanded polyglutamine proteins and is responsible for their ubiquitination and degradation by proteasomes.

EXPERIMENTAL PROCEDURES

Materials—Lactacystin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dbcAMP, and all cell culture reagents were obtained from Sigma. Lipofectamine 2000, Zeocin, G418, ponasterone A, and mouse monoclonal anti-v5 were purchased from Invitrogen. Rabbit polyclonal anti-ubiquitin was from Dako, and mouse monoclonal anti-GFP was from Roche Applied Science. Goat anti-mouse IgG-Cy3 was purchased from Molecular Probes and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Amersham Biosciences.

Expression Plasmids and Stable Cell Lines—The enhanced green fluorescence protein (EGFP) and tNhtt expression constructs, pIND-tNhtt-EGFP-16Q, pIND-tNhtt-150Q, and the generation of the stable cell lines of these constructs have been described previously (12). The construction of plasmids, pEGFP-N1-MJD(f)-20CAG and pEGFP-N1-MJD(f)-130CAG, pEGFP-N1-MJD(t)-20CAG, and pEGFP-N1-MJD(t)-80CAG were described elsewhere (13). The full-length CHIP cDNA was isolated from the total RNA extracted from HeLa cells by reverse transcription-PCR. Construction of full-length and the U-box-deleted CHIP in pcDNA vector with v5 tag were made using PCR.

Cell Culture, Transfection, Cell Viability Assay, and Counting of Aggregates—The wild type mouse neuro2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and the antibiotics penicillin/streptomycin. The stable cell lines (HD 16Q and HD 150Q) were maintained in the

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[1] The abbreviations used are: HD, Huntington's disease; UPP, ubiquitin proteasome pathway; tNhtt; truncated N-terminal huntingtin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; dbcAMP, N^6,2′-*O*-dibutyryl-adenosine-3′:5′-cyclic monophosphate; GFP, green fluorescent protein; EGFP, enhanced GFP; HSC, heat shock cognate.

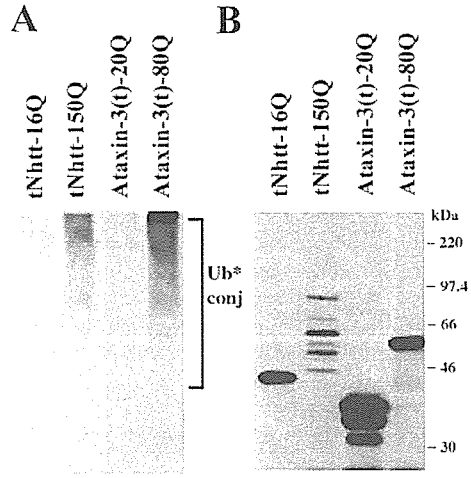

FIG. 1. **Ubiquitination of expanded polyglutamine proteins.** The HD 16Q and HD 150Q cell lines were induced with 1 μM ponasterone A, or the truncated ataxin-3-EGFP fusion constructs with 20Q and 80Q were transiently transfected (1 μg of each/well of 6-well tissue-cultured plate) to the neuro2a cells. Twenty-four hours after induction or transfection, cell lysate were made and subjected to immunoprecipitation as described under "Experimental Procedures." Blots were probed sequentially with ubiquitin antibody (*A*) and GFP antibody (*B*). *Ub * conj*, ubiquitin conjugates.

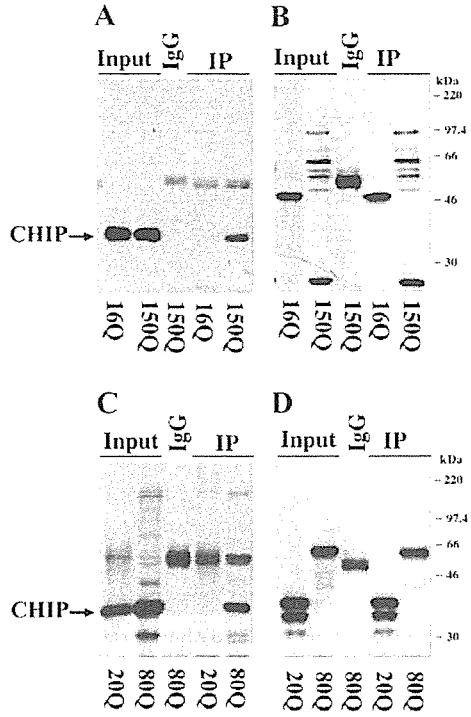

FIG. 2. **Interaction of CHIP with the expanded polyglutamine proteins.** *A* and *B*, the HD 16Q and HD 150Q cell lines were transiently transfected with CHIP (2 μg/well of 6-well tissue-cultured plate), and 12 h after transfection, media were changed, and the cells were induced with 1 μM ponasterone A. Twenty-four hours after induction, cells were collected and processed for immunoprecipitation (*IP*) by anti-GFP. Blots were sequentially probed with anti-v5 (*A*) and anti-GFP (*B*). *C* and *D*, neuro2a cells were first transfected with CHIP (2 μg/well of 6-well tissue-cultured plate). Twelve hours after first transfection, the medium was changed, and the cells were transfected again with the truncated ataxin-3-EGFP fusion constructs (1 μg of each/well) containing 20Q and 80Q. Twenty-four hours after the transfection of ataxin-3 constructs, cell lysates were made and subjected to immunoprecipitation as described in *A* and *B*. The blots were sequentially probed with anti-v5 (*C*) and anti-GFP (*D*).

same medium containing 0.4 mg/ml Zeocin and 0.4 mg/ml G418. One day prior to transfection, cells were plated into 6-well tissue-cultured plates at a subconfluent density. Cells were transiently transfected with expression vectors using Lipofectamine 2000 reagent according to the manufacturer's instruction. Transfection efficiency was ~80–90%. After 24 or 48 h of transfection, cells were used for immunofluorescence staining, co-immunoprecipitation, and immunoblotting. For cell viability assay, cells were first transfected with different expression plasmids. Twelve hours later, cells were harvested and replated into 96-well plates (5×10^3 cells/well). The cells were then differentiated with 5 mM dbcAMP and induced with 1 μM ponasterone A for 3 days. Cell viability was measured by MTT assay as described previously (12). Statistical analysis was performed using Student's *t* test, and $p < 0.05$ was considered to indicate statistical significance. Aggregate formation was manually counted under a fluorescence microscope (~500 transfected cells in each case), and the cells containing more than one aggregate were considered to have a single aggregate.

Co-immunoprecipitation and Immunoblotting Experiment—After 24 or 48 h of transfection, cells were washed with cold phosphate-buffered saline, scraped, pelleted by centrifugation, and lysed on ice for 30 min with radioimmune precipitation assay buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM NaF, 5 mM $Na_4P_2O_7$, 0.1 mM Na_2VO_5, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml Aprotinin). Cell lysates were briefly sonicated, centrifuged for 10 min at 15,000 $\times$ *g* at 4 °C, and the supernatants (total soluble extract) were used for immunoprecipitation as described earlier (9). For each immunoprecipitation experiment, 200 μg of protein in 0.2 ml of radioimmune precipitation assay buffer was incubated either with 5 μl (2 μg) of GFP antibody or 4 μl (2 μg) of normal mouse IgG. Bound proteins were eluted from the beads with SDS (1×) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting according to the procedure described earlier (9). Blot detection was carried out with enhanced chemiluminescence reagent. All primary antibodies were used in 1:1000 dilutions for immunoblotting.

Immunofluorescence Techniques—Cells grown in chamber slides or in 6-well tissue cultured plates were transiently transfected with different constructs. Forty-eight hours after transfection, cells were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 min, washed extensively, then blocked with 5% nonfat dried milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween) for 1 h. Primary antibody (anti-v5, 1:5000 dilutions) incubation was carried out overnight at 4 °C. After several washings with TBST, cells were incubated with Cy3-conjugated secondary antibody (1:500 dilutions) for 1 h, washed several times, and mounted in antifade solution. Samples were observed using a confocal microscope (Fluoview, Olympus), and digital images were assembled using Adobe Photoshop.

Degradation Assay—Neuro2a cells were plated in a 6-well tissue-cultured plate, and on the following day, cells were transiently transfected with full-length ataxin-3 with 20Q and 130Q with or without CHIP. Twenty-four hours post-transfection, cells were chased with 10 μg/ml of cycloheximide for different time periods. Cells collected at each time point were then processed for immunoblotting by anti-GFP.

RESULTS

Misfolded Truncated N-terminal Huntingtin or Ataxin-3 Are Ubiquitinated—We developed several stable neuro2a cell lines in an inducible system, that express tNhtt with normal (16Q) and expanded polyglutamine (150Q) (12). These cell lines were named HD 16Q and HD 150Q, and their corresponding expressed proteins were named tNhtt-16Q and tNhtt-150Q. The cell lines were induced for 1 day with ponasterone A (1 μM) and then processed for immunoprecipitation by anti-GFP. In another experiment, we transfected the truncated ataxin-3 constructs to the neuro2a cell, and after 1 day, cells were collected and processed for immunoprecipitation by GFP antibody. Blots were sequentially probed with anti-ubiquitin and anti-GFP. As shown in Fig. 1*A*, truncated huntingtin with 150Q proteins or the truncated ataxin-3 with 80Q proteins were ubiquitinated, whereas those truncated proteins with normal glutamine repeats were not ubiquitinated. Fig. 1*B* showed the same blot as those in *A* after probing with GFP antibody. The tNhtt-150Q

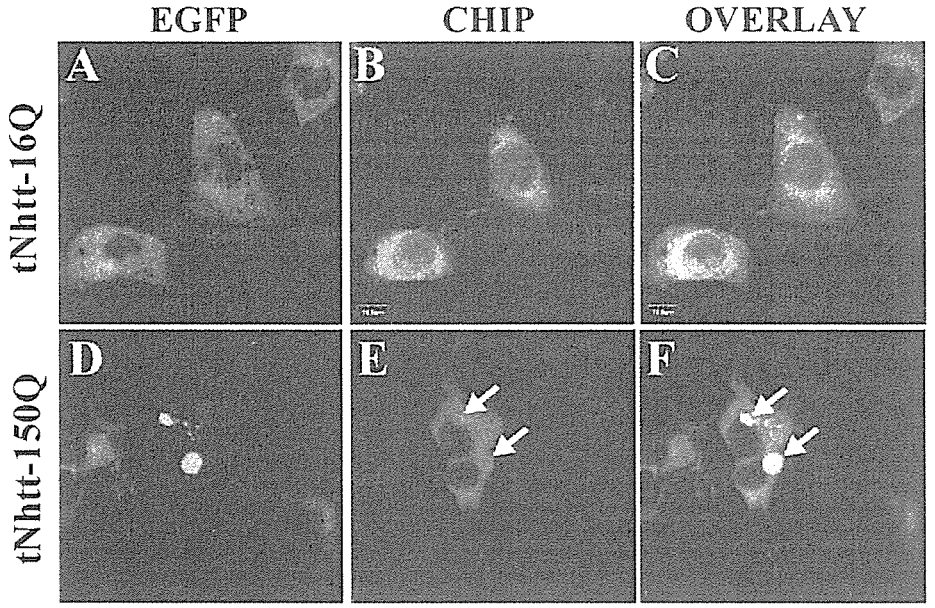

FIG. 3. **Recruitment of CHIP to the mutant huntingtin aggregates.** The HD 16Q (*A–C*) and HD 150Q (*D–F*) cells were transiently transfected with CHIP and induced in the similar way as described in the Fig. 2. Cells were then subjected to immunofluorescence staining with anti-v5. Cy3-conjugated secondary antibody was used to stain the CHIP. *Arrows* indicate the recruitment of CHIP to the huntingtin aggregates.

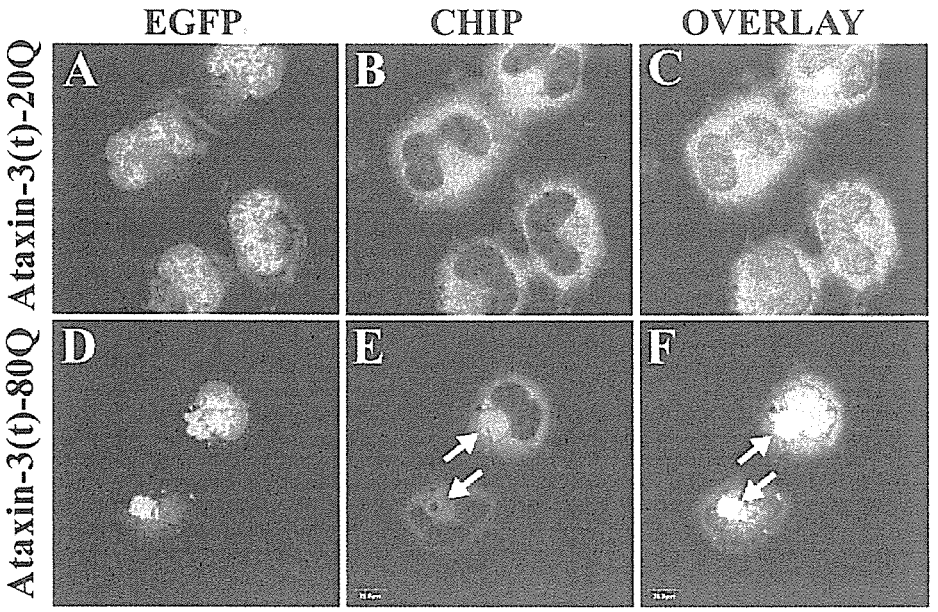

FIG. 4. **CHIP associates with the ataxin-3 aggregates.** The neuro2a cells were sequentially transfected with CHIP and truncated ataxin-3-EGFP fusion constructs containing 20Q (*A–C*) and 80Q (*D–F*). Forty-eight hours later, cells were processed for immunofluorescence staining using v5 antibody. Cy3-conjugated secondary antibody was used to stain the CHIP. *Arrows* indicate the recruitment of CHIP to the ataxin-3 aggregates.

appeared as multiple bands because of the instability of the CAG repeats.

CHIP Interacts with the Polyglutamine-expanded Truncated N-terminal Huntingtin or Ataxin-3—Because misfolding promotes the ubiquitination of the expanded polyglutamine proteins, we next wanted to know the identity of the ubiquitin ligase that is responsible for the misfolding-dependent ubiquitination. We first tested the possibility of CHIP ubiquitin ligase, because recently, CHIP has been shown to be responsible for the ubiquitination and degradation of the misfolded proteins. CHIP was transiently transfected into HD 16Q and HD 150Q cells, the cells were induced with ponasterone A for 1 day, and then the cell lysates were processed for immunoprecipitation by anti-GFP. In a similar experiment, CHIP was co-transfected along with a different truncated ataxin-3 construct, and then the cell lysates were processed for immunoprecipitation. In both experiments, blots were detected with anti-v5 antibody. As shown in Fig. 2, *A* and *C*, CHIP was immunoprecipitated with the truncated N-terminal huntingtin with 150Q and truncated ataxin-3 with 80Q but not the truncated N-terminal huntingtin with 16Q or truncated ataxin-3 with 20Q. Fig. 2, *B* and *D* showed the same blot as in Fig. 2, *A* and *C*, respectively, after detection with anti-GFP.

Association of CHIP with Polyglutamine Aggregates—Next

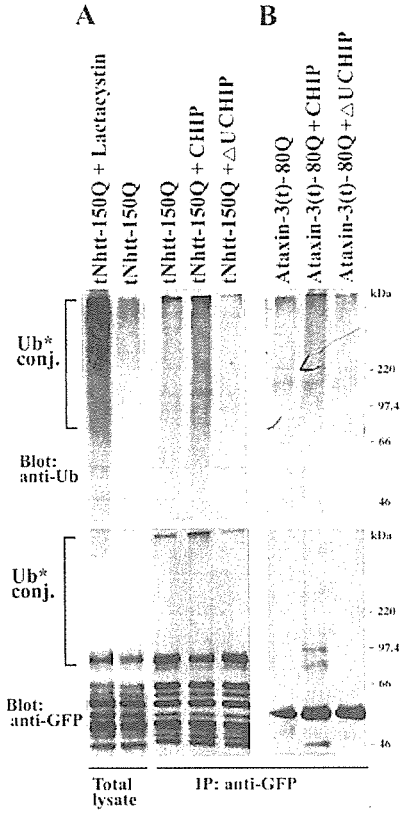

FIG. 5. **Involvement of CHIP in the ubiquitination of expanded polyglutamine proteins.** *A*, the HD 150Q cells were transiently transfected with full-length CHIP, U-box-deleted CHIP or the empty vectors (2 μg of each/well of 6-well tissue-cultured plate) and induced and processed for immunoprecipitation (*IP*) in the similar way as described in the Fig. 2. Blots were sequentially probed with anti-ubiquitin (*top blot*) and anti-GFP (*bottom blot*). In total lysate lanes, induced HD 150Q cells were left untreated or treated with 10 μM lactacystin for 8 h, and then the cell lysate were made and subjected to immunoblotting. *B*, neuro2a cells were first transfected with full-length CHIP, U-box-deleted CHIP, and empty vector (same amounts as used in *A*), and after 12 h, the cells were transfected again with truncated ataxin-3 constructs with 20Q and 80Q in the similar way as described in Fig. 2. The cell lysate were then processed for immunoprecipitation by anti-GFP followed by sequential immunoblotting with anti-ubiquitin (*top blot*) anti-GFP (*bottom blot*). *Ub* conj*, ubiquitin conjugates.

we checked the normal distribution and recruitment of CHIP to the polyglutamine aggregates. First we transiently transfected the CHIP into the HD 16Q and HD 150Q cells, and then the cells were induced to express the truncated huntingtin proteins. After 1 day of induction, cells were processed for immunofluorescence experiments using anti-v5 antibody. CHIP was normally localized into the cytosolic compartment in the wild type neuro2a cells or in the uninduced HD 16Q and HD 150Q cells (Fig. 3). Induction of the expression of the tNhtt-16Q protein did not alter the localization pattern of CHIP in the HD 16Q cell; however, the induction of tNhtt-150Q protein in the HD 150Q cell caused the recruitment of CHIP to the aggregates (Fig. 3). Next, we tested the similar redistribution of CHIP in the ataxin-3 aggregates. CHIP was co-transfected along with truncated ataxin-3 constructs and after 2 days of transfection, cells were processed for immunofluorescence experiments. As expected, CHIP was also recruited to truncated ataxin-3 aggregates (Fig. 4).

CHIP Enhances the Ubiquitination of Polyglutamine-expanded Truncated N-terminal Huntingtin or Ataxin-3—Because CHIP co-immunoprecipitates with expanded polyglutamine proteins and recruits the polyglutamine aggregates, we further tested its possible involvement in the ubiquitination of the expanded polyglutamine proteins. To test this hypothesis, we transfected CHIP (both full-length and U-box-deleted) to the HD 150Q cells, or co-transfected CHIP along with truncated ataxin-3 constructs. The cell lysate were then made and processed for immunoprecipitation by anti-GFP. Fig. 5 showed that CHIP enhanced the rate of ubiquitination of both truncated N-terminal huntingtin containing 150Q (Fig. 5*A*, *top blot*) as well as truncated ataxin-3 with 80Q (Fig. 5*B*, *top blot*). This enhanced rate of ubiquitination was prevented by the deletion of U-box of CHIP. The bottom blots of both Fig. 5, *A* and *B* are the same blots as the top blots, respectively, but probed with anti-GFP. Anti-GFP also detected smears of ubiquitinated derivatives of expanded polyglutamine proteins in the only CHIP-transfected cell lysate. The lactacystin-treated cell lysate was used as positive control to compare the CHIP-induced ubiquitination profile (Fig. 5*A*, *first two lanes*). Because the deletion of U-box of CHIP reduced the rate of ubiquitination, we further tested whether the U-box-deleted CHIP still associates with the polyglutamine aggregates. As shown in Fig. 6, U-box deleted CHIP also recruits to the huntingtin aggregates. We have also observed the association of U-box-deleted CHIP with the

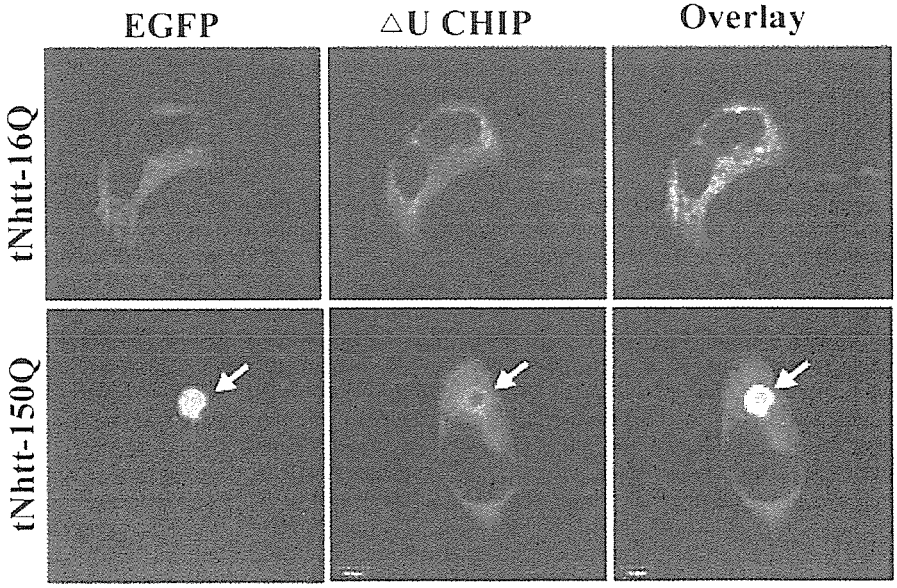

FIG. 6. **Association of U-box-deleted CHIP with the huntingtin aggregates.** The HD 16Q and HD 150Q cells were transiently transfected with U-box-deleted CHIP and induced in a similar way as described in the Fig. 2. Cells were then subjected to immunofluorescence staining with anti-v5. Cy3-conjugated secondary antibody was used to stain the CHIP. *Arrows* indicate the recruitment of U-box deleted CHIP to the huntingtin aggregates.