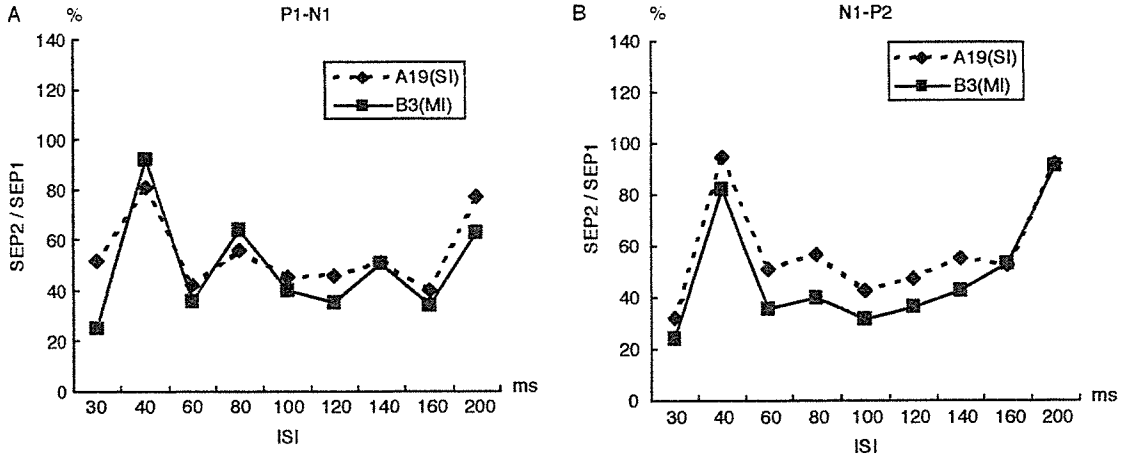

Fig. 3. Recovery curve of P1–N1 (A) and N1–P2 (B) components of the subdurally recorded SEP amplitude in the paired pulse stimulation paradigm. Dotted lines; recovery curve at A19 (SI), solid lines; recovery curve at B3 (MI). The second response was significantly less suppressed at ISIs of 40 and 200 ms, as compared with other ISIs, for P1–N1 and N1–P2 components both at SI and MI.

suppression of SEP 2 at ISIs of 40 and 200 ms (labeled as less suppressed) was significantly different as compared with that of other ISIs (labeled as suppressed) (P1–N1 component: $P=0.004$, N1–P2 component: $P=0.003$).

4. Discussion

With regard to the upper limb stimulation, Cowan et al. recorded intraoperative epicortical giant SEPs under general anesthesia (Cowan et al., 1986). They showed that the latency and polarity of the SEP waveforms, recorded from the cortex apparently posterior to the postcentral gyrus, were similar to those of the scalp recorded ones, although EEG and electrocorticogram (ECoG) were not simultaneously recorded (Cowan et al., 1986). However, there was no previous report of giant SEPs recorded while awake, and furthermore, no studies recorded directly from SI and MI simultaneously. To our knowledge, this is the clear invasive recording of giant SEPs at both SI and MI following lower limb stimulation in an awake patient with cortical reflex myoclonus, and thus it enables us to investigate at least a part of the mechanism underlying generation of cortical reflex myoclonus. However, we should consider two technical limitations in this study. First, some part of the left foot/leg SMI was not covered by the subdural electrodes. Second, the influence of cortical dysplasia or epileptic hyperexcitability on giant SEP generation could not be excluded.

Giant SEPs recorded from SI and MI were similar in morphology. These findings indicate that both SI and MI are hyperexcitable to somatosensory stimuli. The fact that the peaks at SI always occurred earlier than those at MI by about 6 ms suggests that, in the present patient, enhanced response to somatosensory input initially occurred in SI due to its own hyperexcitability, and subsequently the impulse is conducted to MI. The delay of 6 ms seems consistent with cortico-cortical conduction time, since conduction time of a similar duration have been recorded between different muscles within MI in cortical myoclonus (Brown et al., 1991).

Therefore, giant SEPs in this patient are composed of two components; one due to SI hyperexcitability causing giant response to the somatosensory input, and the other arising from MI hyperexcitability most likely induced by the input from SI. This hyperexcitability enhanced by SI, in the present patient, was supported by the fact that SI was the most usual source of the pre-myoclonus spike in patients with cortical reflex myoclonus (Uesaka et al., 1996), but it may not completely exclude the possibility that MI itself is hyperexcitable. Although there is a direct input from the thalamus to the motor cortices and MI alone could also be the source of the pre-myoclonus spike in some patients with cortical myoclonus (Terao et al., 1997; Uesaka et al., 1996), the present findings do not suggest the role of this system at least in the present case. Previous magnetoencephalographic (MEG) study on cortical reflex myoclonus suggested the hyperexcitability of both SI and MI, but the time difference between SI and MI was not clearly delineated (Mima et al., 1998). It may be explained by the limited sensitivity of MEG as compared with epicortical recording or by the different etiology between the two disease groups.

The similar morphology of early components of giant SEPs between scalp and epicortical recording is consistent with the previous upper limb study (Cowan et al., 1986). However, the peaks P1 and N1 occurred consistently earlier at subdural SI than over the scalp by 4.3–11.1 ms. It could be explained, at least partly, by the phase shift of about 8 ms as the effect of skull bone which could behave as the 15 Hz high frequency filter (Tyner et al., 1983). On the other hand, P1 and N1 peaks at subdural MI were similar to those at scalp recording. Foot MI was buried in the mesial surface whereas foot SI was exposed both on the lateral and mesial surfaces as shown in Fig. 1C. Taken together with the skull bone effect as described above, it is postulated that scalp recorded P1 and N1 reflect those arising from SI rather than MI in the present

patient. In contrast, the morphology of the late components (including P2) of giant SEPs was not identical between the scalp EEG and the ECoG. It may be explained by the different position of reference electrodes and different ISI employed between the scalp and ECoG recording (the former adopted linked earlobes as the reference and ISI of 2.9 s, and the latter left mastoid process and 1 s).

The similarity in latencies between SEPs and SEFs was also observed in previous study (Mima et al., 1998). This result could be partly explained by the characteristics of MEG because MEG signals usually reflect the cortical activities that are derived from the intracellular current tangentially oriented to the scalp.

Kakigi and Shibasaki reported giant SEPs in response to tibial nerve stimulation in 3 patients with cortical reflex myoclonus due to various causes (progressive myoclonic epilepsy, sialidosis and uremic encephalopathy) (Kakigi and Shibasaki, 1987b). They showed enhanced P1–N1 components localized maximum at Cz or CPz. Their findings are consistent with our scalp SEPs in terms of amplitude. In their study, one patient showed delayed P1 and N1 components, as seen in our patient.

Paired SEPs demonstrated that the SEP2 was less suppressed, as compared with other ISIs, both at SI and MI for the ISIs of 40 and 200 ms. Decreased inhibition around ISI 40 ms was similar to the previous upper limb studies (Shibasaki et al., 1985; Ugawa et al., 1991). This consistent result between upper and lower limb giant SEPs indicates the mechanism of disinhibition at ISI of 40 ms which could be explained by the intracortical factors after the somatosensory afferent signals reach the SI. It could correlate with exaggerated scalp recorded 16–20 Hz (interval of 42–50 ms) oscillatory EEG potentials over the contralateral SMI in patients with positive or negative myoclonus (Ugawa et al., 2003). On the other hand, disinhibition at ISI of 200 ms could be explained by the earlier recovery usually almost completed at ISI of 300 ms in normal subjects (Ugawa et al., 1991). SEP2 was suppressed by the preceding stimulus (SEP1) to a lesser degree, as compared with other ISIs, but it was still less than 100% of the SEP1 at ISIs of 40 and 200 ms, as opposed to more than 100% of SEP1 in the previous studies (Shibasaki et al., 1985; Ugawa et al., 1991). It may be explained by the fact that the present patient was treated by many kinds of anticonvulsants that could enhance inhibitory activity within the brain. The effect of anticonvulsants and epileptic activity on the result of single pulse as well as paired pulse SEPs remains to be taken into consideration.

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Structure of human ubiquitin-conjugating enzyme E2 G2 (UBE2G2/UBC7)

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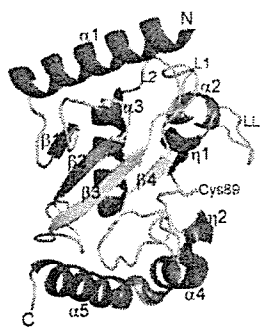

The human ubiquitin-conjugating enzyme E2 G2 (UBE2G2/UBC7) is involved in protein degradation, including a process known as endoplasmic reticulum-associated degradation (ERAD). The crystal structure of human UBE2G2/UBC7 was solved at 2.56 Å resolution. The UBE2G2 structure comprises a single domain consisting of an antiparallel β-sheet with four strands, five α-helices and two 3_{10}-helices. Structural comparison of human UBE2G2 with yeast Ubc7 indicated that the overall structures are similar except for the long loop region and the C-terminal helix. Superimposition of UBE2G2 on UbcH7 in a c-Cbl–UbcH7–ZAP70 ternary complex suggested that the two loop regions of UBE2G2 interact with the RING domain in a similar way to UbcH7. In addition, the extra loop region of UBE2G2 may interact with the RING domain or its neighbouring region and may be involved in the binding specificity and stability.

1. Introduction

Ubiquitin-dependent protein degradation plays an important role in the regulation of various cellular processes, including cell-cycle progression, signal transduction, transcription, DNA repair and protein quality control (Koepp *et al.*, 1999; Laney & Hochstrasser, 1999). Ubiquitination involves the successive actions of the ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase enzymes (E3) (Hershko & Ciechanover, 1998; Pickart, 2001). The E1 enzyme activates free ubiquitin and transfers it to E2 through a thioester linkage between the ubiquitin C-terminus and an E2 active-site cysteine. The E3 enzyme recognizes its substrate and E2 and catalyzes the formation of an isopeptide bond between a lysine ε-amino group of the substrate (or ubiquitin) and the C-terminal carboxyl group of ubiquitin Gly76. Over 30 human E2s have been identified and they all contain a conserved ~150 amino-acid catalytic core. The E2 enzymes are grouped into four classes depending on the presence and the location of additional sequences (Jentsch, 1992). Some of these enzymes contain extra C-terminal and/or N-terminal extensions from the core domain. The class I enzymes are the smallest E2 enzymes and consist almost entirely of the conserved core domain. The class II enzymes contain an extra C-terminal extension from the core domain, while class III enzymes have an N-terminal extension. The class IV enzymes contain both N- and C- terminal extensions.

The human UBE2G2 gene encodes the ubiquitin-conjugating enzyme E2 G2 (UBE2G2/UBC7), with a molecular weight of 18.6 kDa (165 amino acids). It was mapped to the region of human chromosome 21q22.3 and its transcripts are ubiquitously expressed in human tissues (Katsanis & Fisher, 1998; Rose *et al.*, 1998). Human UBE2G2 is a class I E2 enzyme. Recently, bacterial expression of His-tagged human UBE2G2 was reported (Reyes *et al.*, 2006). The human UBE2G2 protein shares 100, 62, 47 and 27% identities to murine UBE2G2/UBC7 (MmUBC7), yeast Ubc7, human UBE2G1 and human UbcH7, respectively (Fig. 1*a*). The crystal structures of yeast Ubc7 (Cook *et al.*, 1997), a human E6AP–UbcH7 complex (Huang *et*

al., 1999) and a human c-Cbl–UbcH7–ZAP-70 complex (Zheng *et al.*, 2000) have been reported. Functional studies have associated yeast Ubc7 and MmUBC7 with the degradation of endoplasmic reticulum (ER) substrates, a process known as ER-associated degradation (ERAD; Jungmann *et al.*, 1993; Fang *et al.*, 2001; Tiwari & Weissman, 2001). Parkin, a gene product responsible for autosomal recessive juvenile Parkinsonism (AR-JP), interacts with human UBE2G2/UBC7 and UBC6 through its RING domain and specifically ubiquitinates the Pael receptor in the presence of the E2s (Imai *et al.*, 2001). Furthermore, exogenous MmUBC7 mediates the ubiquitination and down regulation of both the inositol 1,4,5-triphosphate receptor in human neuroblastoma cells (Webster *et al.*, 2003) and the human type 2 iodothyronine selenodeiodinase (Kim *et al.*, 2003). Recently, the interactions of human UBE2G2/UBC7 with some RING-finger E3s, such as human HRD1 (Kikkert *et al.*, 2004) and TEB4 (Hassink *et al.*, 2005), have been reported. To analyze the structural and functional details of human UBE2G2/UBC7, which is involved in important cellular processes, its structure must be determined and compared with those of its homologues. Here, we report the crystal structure of human UBE2G2/UBC7 at 2.56 Å resolution and discuss its structural aspects.

2. Materials and methods

2.1. Protein expression and purification

The human UBE2G2 gene (Imai *et al.*, 2001) encoding human ubiquitin-conjugating enzyme E2 G2 (UBE2G2/UBC7) was cloned into a modified pENTR vector with a tobacco etch virus (TEV) protease cleavage site, derived from pENTR1A (Invitrogen). The expression vector pET/cMBP-UBE2G2 was constructed using Gateway technology (Invitrogen) with pENTR/TEV-UBE2G2 and pET/cMBP-GATEWAY bearing a T7 promoter, an N-terminal maltose-binding protein (MBP) tag and a Gateway reading frame cassette A (Invitrogen). The UBE2G2 protein was expressed as a fusion with an N-terminal MBP tag and a TEV protease cleavage site in *Escherichia coli* BL21(DE3). The protein was first purified on an amylose-resin column (New England Biolabs) and the MBP tag was then cleaved by His-tagged TEV protease, which was removed using a HisTrap column (GE Healthcare). The protein was purified further by Mono-Q and Superdex 75 column (GE Healthcare) chromatography steps. The yield of purified UBE2G2 protein was 8 mg per litre of culture. The construct that was used for crystallization contained the cloning artifact sequence GGSEF at the N-terminus.

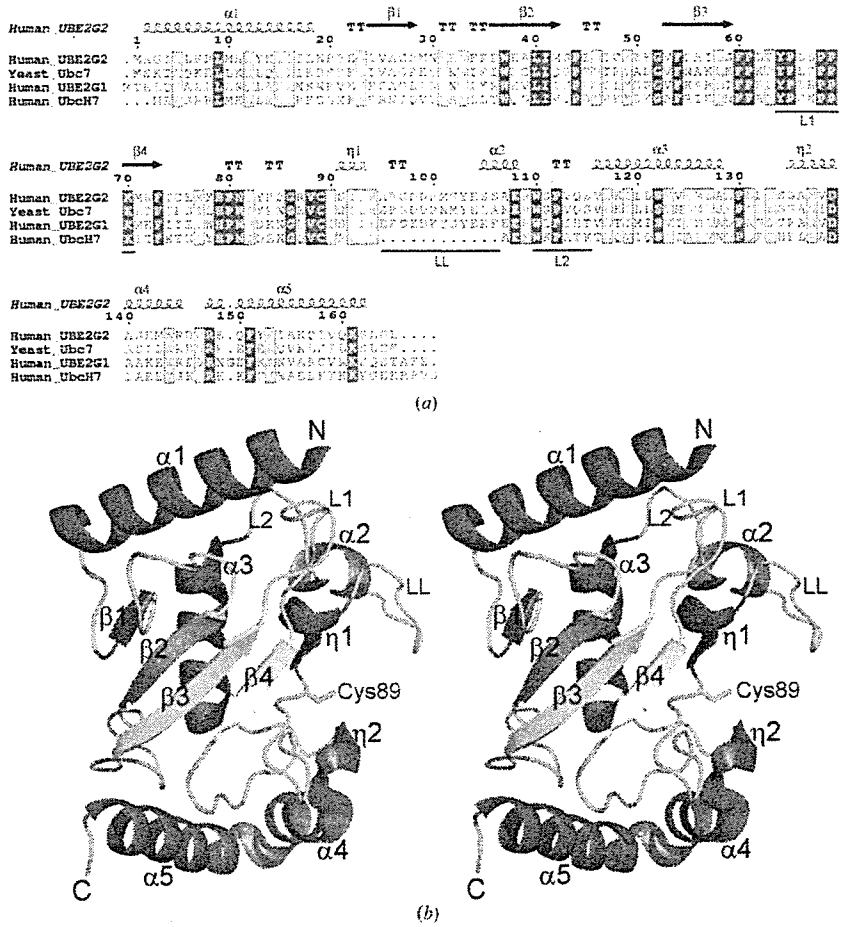

Figure 1
(*a*) Sequence alignment of homologues of human UBE2G2/UBC7. The alignment was generated by *ESPript* (Gouet *et al.*, 1999) with *CLUSTAL W* (Thompson *et al.*, 1994). The secondary structures of the human UBE2G2 protein, as determined by *DSSP* (Kabsch & Sander, 1983), are shown above the sequences (α, α-helix; β, β-strand; η, 3_{10}-helix; TT, β-turn). (*b*) Ribbon representation of the human UBE2G2/UBC7 structure (amino acids 1–165; stereoview). The helices and the β-strands are shown in red and yellow, respectively. The active-site residue (Cys89) is shown as a stick model.

2.2. Crystallization and data collection

Preliminary crystals of human UBE2G2 were obtained under condition No. 42 (0.1 *M* Tris–HCl buffer pH 8.5 containing 1.5 *M* ammonium sulfate and 12% glycerol) of the Crystal Screen 2 crystal screening kit (Hampton Research) using the 96-well sitting-drop vapour-diffusion method. The crystals of UBE2G2 used for structure determination were obtained in drops composed of 1 µl 8.5 mg ml^{-1} protein solution (20 m*M* Tris–HCl buffer pH 8.0 containing 120 m*M* NaCl, 2 m*M* DTT) and 1 µl reservoir solution (0.1 *M* Tris–HCl buffer pH 8.1 containing 1.45 *M* ammonium sulfate and 12% glycerol; Hampton Research) by the hanging-drop vapour-diffusion method against 500 µl reservoir solution. A rod-like crystal (~350 × 100 × 100 µm) was obtained within a few days and was used for data collection. The data collection was carried out at 100 K, with the reservoir solution containing 27.5% glycerol as a cryoprotectant. The diffraction data were collected at SPring-8 BL26B1 (Yamamoto *et al.*, 2002) and were recorded on a Jupiter 210 CCD detector (Rigaku). All diffraction data were processed with the *HKL*2000 program suite (Otwinowski & Minor, 1997).

2.3. Structure determination and refinement

The structure was solved by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 1997) with the yeast Ubc7 structure (PDB code 2ucz; Cook *et al.*, 1997) as a search model. Data in the resolution range 50–3.0 Å were used in both rotation and translation calculations, which gave an obvious solution with significant contrast, resulting in three molecules in the asymmetric unit with a Matthews coefficient (V_M) of 3.83 Å^3 Da^{-1} and a solvent content of 67.91%. The model was corrected iteratively using *O* (Jones *et al.*, 1991) and was refined to 2.56 Å using *LAFIRE* (Yao *et al.*, 2006), *REFMAC*5 (Murshudov *et al.*, 1997) and *Crystallography & NMR System* (*CNS*; Brünger *et al.*, 1998). The crystallographic data and refinement statistics are presented in Table 1. Since there was additional electron density, four residues of the cloning artifact sequence at the N-terminus were also modelled. The final model includes 507 amino-acid residues of three UBE2G2 monomers and 23 water molecules in the asymmetric unit. In the loop regions (residues 100–106 and 131–133), the electron density corresponding to the side chains was ambiguous, which increased the *B* factor. In addition, relatively large areas of the molecular surface were exposed to the solvent in the crystal as the solvent content was high. These features resulted in the high average *B* factor. The quality of the model was inspected using *PROCHECK* (Laskowski *et al.*, 1993). The figures were created using *PyMOL* (DeLano, 2005).

Table 1
X-ray data-collection and refinement statistics.

Values in parentheses are for the outer shell (2.65–2.56 Å).

Data collection	
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 63.52, b = 87.61, c = 157.41
Wavelength (Å)	1.000
Resolution (Å)	50–2.56
Total reflections	117935
Unique reflections	28705
Redundancy	4.1 (3.7)
Completeness (%)	97.5 (84.6)
$I/\sigma(I)$	22.2 (4.2)
R_{sym}† (%)	5.5 (29.3)
Refinement	
Resolution (Å)	49.43–2.56
No. of reflections	28395
No. of protein atoms	3996
No. of water molecules	23
R_{work} (%)	22.8
R_{free}‡ (%)	26.2
R.m.s.d. bond lengths (Å)	0.009
R.m.s.d. bond angles (°)	1.6
Average *B* factor (Å^2)	75.7
Ramachandran plot	
Most favoured regions (%)	85.9
Additional allowed regions (%)	14.1
Generously allowed regions (%)	0.0
Disallowed regions (%)	0.0

† $R_{sym} = \sum|I_i - I_{avg}|/\sum I_i$, where I_i is the observed intensity and I_{avg} is the average intensity. ‡ R_{free} is calculated for 10% of randomly selected reflections excluded from refinement.

3. Results and discussion

The crystal structure of human UBE2G2 comprises a single domain consisting of an antiparallel β-sheet with four strands (β1–β4), five α-helices (α1–α5) and two 3_{10}-helices (η1 and η2; Fig. 1*b*). The ubiquitin-accepting residue Cys89 is located near η1. The overall folding of UBE2G2 corresponds to the typical fold of ubiquitin-conjugating enzymes. According to analytical ultracentrifugation, the

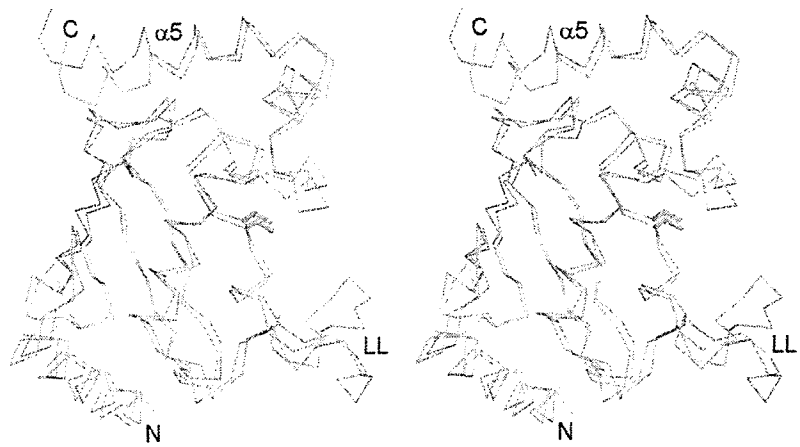

Figure 2
Superimposition of the main-chain structures of human UBE2G2 (green) and yeast Ubc7 (orange) (PDB code 2ucz; Cook *et al.*, 1997) (stereoview). The active-site cysteine residues are shown as stick models. The superimposition was carried out with *LSQKAB* (Kabsch, 1976).

molecular weight of UBE2G2 was ~18 kDa (data not shown), indicating that the UBE2G2 protein exists as a monomer in solution.

Fig. 2 shows the superimposition of the main-chain structures of human UBE2G2 and yeast Ubc7 (Cook *et al.*, 1997). The overall structure of UBE2G2 is remarkably similar to that of yeast Ubc7 (r.m.s.d. = 2.15 Å over 164 C^{α} atoms). The major differences between human UBE2G2 and yeast Ubc7 are the structure of the long loop (LL) region (95–106) and the angle of the C-terminal helix. The C-terminal helix (α5) of UBE2G2 is closer to the β-sheet core region than that of yeast Ubc7. The important interactions of UBE2G2 in the contact region of the C-terminal helix and the core region are the hydrophobic interactions among Phe54, Met77, Phe78, Ile154 and Ile158 and the salt bridge between Glu76 and Lys161. The residues Glu76, Ile154 and Ile158 are replaced with Ser76, Gln154 and Ser158 in yeast Ubc7, respectively, suggesting that the interactions of yeast Ubc7 are weaker than those of UBE2G2. Consequently, the angle of the C-terminal helix (α5) may change. Recently, the crystal structure of the human ubiquitin-conjugating enzyme E2 G1 (UBE2G1), which is another human homologue of yeast Ubc7, was deposited in the PDB (PDB code 2awf). A structural comparison of UBE2G2 with UBE2G1 revealed that the overall folding of UBE2G2 is similar to that of UBE2G1 (r.m.s.d. = 1.12 Å over 115 C^{α} atoms), but in UBE2G1 the residues 98–106 within the long loop (LL) region and the C-terminal helices (η2, α4 and α5) were not located in the model owing to disorder.

Zheng and coworkers reported the crystal structure of a c-Cbl–UbcH7–ZAP70 peptide ternary complex (PDB code 1fbv; Zheng *et al.*, 2000). It revealed how the RING domain of c-Cbl recruits the ubiquitin-conjugating enzyme UbcH7. Fig. 3(*a*) shows the superimposition of the main-chain structures of UBE2G2 and UbcH7 in the ternary complex. The overall folding of UBE2G2 and UbcH7 overlaps roughly (r.m.s.d. = 2.95 Å over 143 C^{α} atoms). Fig. 3(*b*) shows a close-up view of the interface between the RING domain and the E2s. The critical residues of UbcH7 for the interaction with the RING domain, Pro62, Phe63, Lys96, Pro97 and Ala98 (Zheng *et al.*, 2000), and the corresponding residues of UBE2G2, Pro65, Leu66,

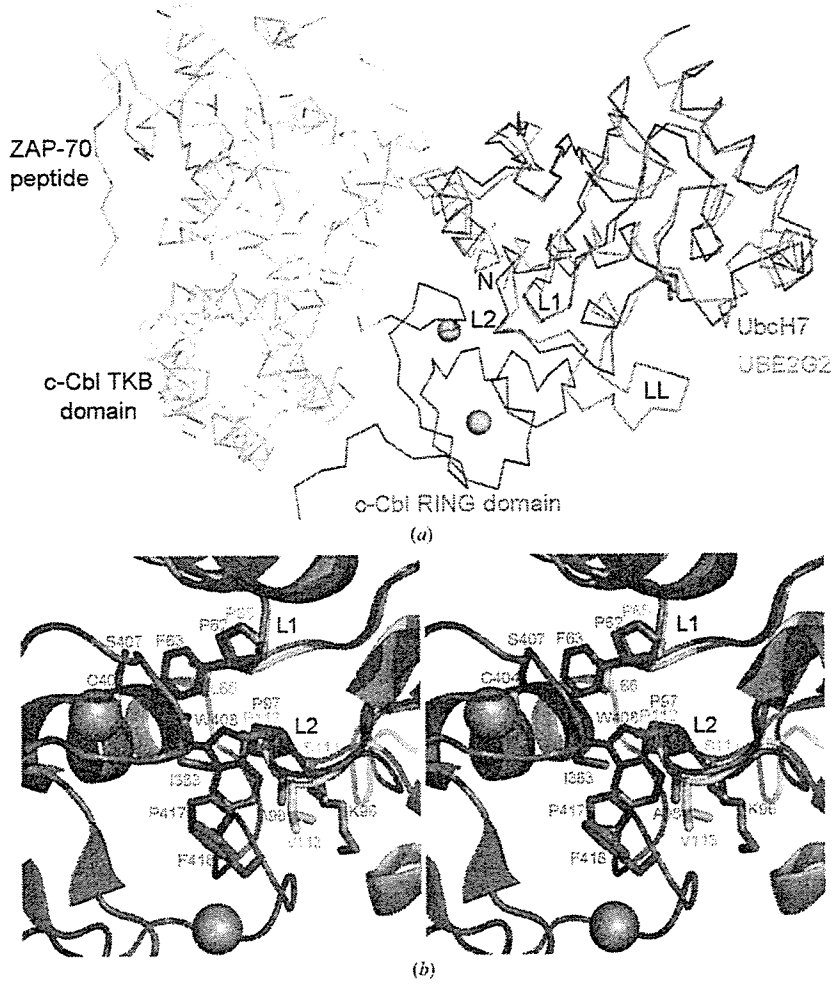

Figure 3
(*a*) Superimposition of the main-chain structures of human UBE2G2 and UbcH7 in the c-Cbl–UbcH7–ZAP70 peptide ternary complex (PDB code 1fbv; Zheng *et al.*, 2000). The TKB domain and linker sequence of c-Cbl, the RING domain of c-Cbl, the ZAP-70 peptide and human UbcH7 are coloured yellow, red, cyan and magenta, respectively. The zinc ions are indicated by grey spheres. The human UBE2G2 protein is coloured green. The active-site cysteine residues are shown as stick models. (*b*) Close-up view of the ribbon representation of the interface between the RING domain and UbcH7 in the c-Cbl–UbcH7–ZAP70 complex and the superimposition of UBE2G2 on UbcH7 (stereoview). The colouring is the same as that in Fig. 3(*a*). The critical residues for the interaction of UbcH7 with the *RING* domain and the corresponding residues of UBE2G2 are shown as stick models. All superimpositions were carried out with *LSQKAB* (Kabsch, 1976).

Ser111, Pro112 and Val113, overlap remarkably well (r.m.s.d. = 0.768 Å over five C^{α} atoms), suggesting that the L1 (64–70) and L2 (110–115) loops of UBE2G2 are involved in the interaction with the RING domain in a similar way as UbcH7. This is consistent with the previous results that Parkin binds to UBE2G2 as well as UbcH7 and ubiquitinates substrates (Imai *et al.*, 2000, 2001). In addition, UBE2G2 has the extra long loop (LL) region (95–106), which is probably located on the side near the RING domain. The *B* factor of the LL region is relatively high, implying the possibility of conformational flexibility. The LL region may interact with the RING domain or its neighbouring region and may be involved in the binding specificity and stability.

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ORIGINAL PAPER

α-Synuclein is colocalized with 14-3-3 and synphilin-1 in A53T transgenic mice

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Abstract α-Synuclein is a major constituent of Lewy bodies, the neuropathological hallmark of Parkinson's disease (PD). Three types of α-synuclein mutations, A53T, A30P, and E46K, have been reported in familial PD. Wild-type α-synuclein accumulates at high concentrations in Lewy bodies, and this process is accelerated with mutated A53T α-synuclein. The accumulation of α-synuclein is thought to be toxic, and causes neuronal death when α-synuclein aggregates into protofibrils and fibrils. Lewy bodies contain not only α-synuclein, but also other proteins including 14-3-3 proteins and synphilin-1. 14-3-3 Proteins exist mainly as dimers and are related to intracellular signal transduction pathways. Synphilin-1 is known to interact with α-synuclein, promoting the formation of cytoplasmic inclusions like Lewy bodies in vitro. To investigate the colocalization of α-synuclein, synphilin-1, and 14-3-3 proteins, we performed immunohistochemical studies on α-synuclein, 14-3-3 proteins, and synphilin-1 in the brain and spinal cord of A53T transgenic mice. In homozygous mouse brains, α-synuclein immunoreactivity was observed in the neuronal somata and processes in the medial part of the brainstem, deep cerebellar nuclei, and spinal cord. The distribution of 14-3-3 proteins and synphilin-1 immunoreactivity was similar to that of α-synuclein in the homozygous mice. Double immunofluorescent staining showed that α-synuclein and synphilin-1 or 14-3-3 proteins were colocalized in the pons and spinal cord. These results indicate that the accumulation of mutant α-synuclein occurs in association with 14-3-3 proteins and synphilin-1, and may cause the sequestration of important proteins including 14-3-3 proteins and synphilin-1. The sequestration and subsequent decrease in 14-3-3 proteins and synphilin-1 levels may account for neuronal cell death.

Keywords α-Synuclein · A53T transgenic mice · Parkinson's disease · 14-3-3 Proteins · Synphilin-1 · Immunohistochemistry

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Introduction

Parkinson's disease (PD), one of the most common neurodegenerative diseases, is characterized neuropathologically by the presence of intracytoplasmic inclusions called Lewy bodies (LBs), which mainly contain aggregated α-synuclein [34]. Point mutations in the α-*Synuclein* genes have been discovered in families afflicted with autosomal dominant inherited PD [23, 28, 38]. α-Synuclein is expressed predominantly in the presynaptic nerve terminals [13]. Under normal conditions, it is thought to have a role in the modulation of synaptic vesicle turnover and synaptic plasticity [6, 8]. However, this physiological role of α-synuclein is not essential for nerve terminal function, because α-synuclein knockout mice display only a mild phenotype. Nevertheless, α-synuclein is likely to protect nerve terminals under

unusual conditions such as neuronal stress or injuries, since the transgenic expression of α-synuclein has been shown to abolish the lethality and neurodegeneration caused by the deletion of cysteine-string protein-α (CSPα), a co-chaperone protein localized in the synaptic vesicles [6]. Therefore, the loss of functional α-synuclein may predispose dopaminergic neurons to oxidative injury or mitochondrial dysfunction.

Lewy bodies also contain other proteins including ubiquitin [25], parkin [33], cytoskeletal proteins like neurofilaments [32], and septins [16], 14-3-3 proteins [22], and synphilin-1 [36]. The 14-3-3 proteins, a family of protein chaperones, are abundant in the brain, comprising approximately 1% of the total brain proteins [5]. 14-3-3 proteins consist of seven different isoforms, named with Greek letters (β, ε, γ, η, σ, θ, ζ) [12]. 14-3-3 proteins exist mainly as homo- or hetero-dimers consisting of ε and ζ, or θ and ζ subunits, and participate in intracellular signal transduction pathways [1].

14-3-3 Proteins are increased in the cerebrospinal fluid from patients with Creutzfeldt-Jakob disease (CJD), and the detection of 14-3-3 proteins is a marker in the pre-mortem diagnosis of CJD [15]. In recent studies, 14-3-3 proteins were found in abnormal pathological structures, including the neurofibrillary tangles in Alzheimer's disease [24], the Pick bodies in Pick's disease [35], the Lewy bodies in Parkinson's disease [22], the Lewy body-like hyaline inclusions in amyotrophic lateral sclerosis [21], the glial cytoplasmic inclusions in multiple system atrophy [20], the nuclear inclusions in spinocerebellar ataxia-1 [7], the prion plaques in sporadic CJD, and the florid plaques in variant CJD [30]. Ostrerova et al. [27] showed that regions of α-synuclein and 14-3-3 proteins share over 40% homology, and bind to each other. These proteins were found to oppositely regulate parkin activity [31], suggesting important roles for 14-3-3 and α-synuclein together with parkin in the pathogenesis of PD.

On the other hand, Engelender et al. [10] identified synphilin-1, a novel protein which also interacts with α-synuclein to form cytoplasmic inclusions in vitro. The C-terminus of synphilin-1 is closely associated with the C-terminus of α-synuclein [19]. The function of synphilin-1 is not fully understood yet; however, it is enriched in the presynaptic terminals, possibly mediating the synaptic function attributed to α-synuclein [29]. Synphilin-1 is enriched in the central cores of LBs, and is presumed to play a role in LB formation in vivo [36]. Recently, Eyal et al. [11] identified synphilin-1A, an isoform of synphilin-1, which has enhanced aggregatory properties and causes neurotoxicity. Synphilin-1A is also observed in LBs. Synphilin-1 and synphilin-1A differ in their exon organization, and are translated from different start codons. Therefore, the N-terminus of synphilin-1A is different from that of synphilin-1. In addition, a mutation of the synphilin-1 gene has been detected in two German PD patients [26]. Collectively, clarifying the roles of α-synuclein, 14-3-3, and synphilin-1 in the process of LB formation may shed some light on the pathogenesis of PD.

In this study, we examined A53T-Tg mice, which overexpress mutated human A53T α-synuclein under the control of a prion promoter, using immunohistochemistry for α-synuclein, the seven 14-3-3 isoforms, and synphilin-1. α-Synuclein has a tendency to self-aggregate and form fibrils in the presence of the familial PD-linked A53T mutation of α-synuclein [9], and the A53T-Tg mice show numerous α-synuclein-based aggregates in the brain [14]. We determined the distribution of the seven 14-3-3 isoforms and synphilin-1 in the aggregates from these mice to uncover the roles of 14-3-3 proteins and synphilin-1 in the pathogenesis of PD.

Materials and methods

Transgenic mice expressing A53T mutant human α-synuclein (A53T-Tg mice)

We used transgenic mice expressing the A53T mutant human α-synuclein (A53T-Tg mice), which were described in a previous paper [14]. In brief, these Tg mice were generated by using the MoPrP. Xho expression vector, which drives the high expression of A53T mutant human α-synuclein in most CNS neurons. Homozygous A53T-Tg mice develop a motor phenotype, but their litter heterozygous A53T-Tg mice show no neurological symptoms or signs, at least before they become 20 months old. We investigated five homozygous A53T-Tg mice and five heterozygous A53T-Tg mice.

Tissues

The A53T-Tg mice were deeply anesthetized with sodium pentobarbital and then perfused transcardially with 0.01 M phosphate-buffered saline (PBS; Nacalai Tesque, Kyoto, Japan), followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). Following the surgical removal of the brains and spinal cords, the tissues were fixed for 24 h in the same fixatives, and then stored in 20% sucrose in 0.1 M PBS (pH 7.4). The brains and spinal cords were sliced into coronal and axial sections, respectively (20 μm thick) on a cryostat.

Primary antibodies

As the primary antibodies, we used a goat polyclonal anti-α-synuclein antiserum [C-20; Santa Cruz Biotechnology (SCB), diluted 1:500], a rabbit polyclonal anti-α-synuclein antiserum (C-20R; SCB, diluted 1:500), a rabbit polyclonal anti-human synphilin-1 antiserum (Sy-1-C; an antibody to the C-terminal region of synphilin-1, 1:100) [17], a rabbit polyclonal anti-ubiquitin antiserum (U5379; SIGMA, diluted 1:1,000), and several types of anti-14-3-3 antibodies: a rabbit polyclonal anti-14-3-3β antiserum (C-20; SCB, diluted 1:2,000), a goat polyclonal anti-14-3-3β antiserum (A-15; SCB, diluted 1:1,000), a rabbit polyclonal anti-14-3-3γ antiserum (C-16; SCB, diluted 1:2,000), a rabbit polyclonal anti-14-3-3ζ antiserum (C-16; SCB, diluted 1:2,000), a rabbit polyclonal anti-14-3-3θ antiserum (C-17; SCB, diluted 1:2,000), a rabbit polyclonal anti-14-3-3ε antiserum (T-16; SCB, diluted 1:400), a goat polyclonal anti-14-3-3η antiserum (E-12:SCB, diluted 1:400), a goat polyclonal anti-14-3-3σ antiserum (C-18, against the C-terminus of 14-3-3σ; SCB, diluted 1:400), and a goat polyclonal anti-14-3-3σ antiserum (N-14, against the N-terminus of 14-3-3σ; SCB, diluted 1:400).

Immunohistochemistry

The brain and spinal cord sections were incubated with the primary antibodies in 0.1 M PBS overnight at 4 C. Subsequently, these sections were treated with the appropriate biotinylated secondary antibodies (diluted 1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature, followed by an incubation with an avidin–biotin–peroxidase complex (ABC) kit (Vector) diluted in 0.1 M PBS (1:200) for 1 h at room temperature. The sections were visualized with 0.01% diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto, Japan), and 0.005% H_2O_2 in 0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Adjoining sections were used for the immunohistochemical investigation of α-synuclein, synphilin-1, and 14-3-3 proteins in the brain and the spinal cord.

Double labeling immunohistochemistry

To investigate the relationship between α-synuclein and 14-3-3 proteins or synphilin-1, the brain sections were incubated with primary antibodies raised against α-synuclein and 14-3-3 or synphilin-1, followed by immunofluorescent staining procedures with fluorescein isothiocyanate-conjugated swine anti-goat immunoglobulins (ACI0408; Biosource) and tetramethylrhodamine-conjugated swine anti-rabbit immunoglobulins (R0156; DAKO).

Results

Distribution of α-synuclein immunoreactivity

In the homozygous mouse brains, α-synuclein immunoreactivity was observed in the neuronal somata and processes. Some α-synuclein immunoreactivity in the somatodendritic compartment appeared as LB-like inclusions. These α-synuclein immunoreactive granules were abundant in the medial part of the midbrain and pons (Figs. 1a, 4), deep cerebellar nuclei (DCN) (Fig. 1b), and spinal cord (Fig. 1c). In these areas, a similar immunolabeling pattern was also observed for ubiquitin (Fig. 1e–g). In age-matched litter heterozygous mouse brains, however, immunostaining for α-synuclein was observed in the neuropil, without any immunostaining in the somatodendritic compartment (Fig. 1d).

Synphilin-1 immunoreactivity

Immunoreactivity for synphilin-1 in the homozygous mice was observed in the somatodendritic compartment of the pons (Fig. 2a), DCN (Fig. 2b) and spinal cord (Fig. 2c). There was no immunoreactivity for synphilin-1 in the axons. In the heterozygous mouse brains, no or faint immunoreactivity for synphilin-1 was detected (Fig. 2d).

14-3-3 Protein immunoreactivity

In the homozygous mice, immunoreactivity for 14-3-3ζ was observed mainly in the somatodendritic compartment in the medial part of the brainstem, motor cortex, and caudoputamen; however, faint immunoreactivity was detected in the spinal cord. 14-3-3ζ immunoreactivity in the heterozygous mice was observed mainly in the cerebral cortex and caudoputamen, but was not detected in the pons (Fig. 2e). Immunoreactivity for 14-3-3β, γ, and θ in the homozygous mouse brains was found mainly in the somatodendritic compartment of the pons, and spinal cord (Fig. 2f, g). In the heterozygous mice, immunoreactivity for 14-3-3β (C-20), γ, and θ was positive in the cerebral cortex, pons, and deep cerebellar nucleus, but there was no or faint immunoreactivity in the spinal cord (Fig. 2h). In the heterozygous mice, immunoreactivity for 14-3-3β (A-15) was also observed in the cerebral cortex, pons, deep cerebellar nucleus, and spinal cord. However, in the

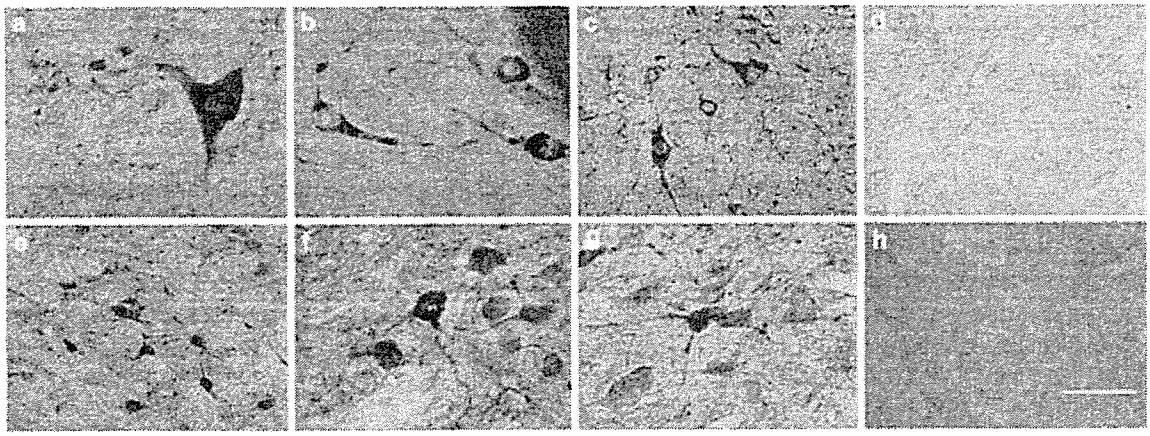

Fig. 1 Immunostaining for α-synuclein (**a–d**) and ubiquitin (**e–h**) in homozygous mice (**a–c**, **e–g**) and heterozygous mice (**d**, **h**). The photomicrographs were taken from the pons (**a**, **e**), deep cerebellar nucleus (**b**, **f**), and cervical spinal cord (**c**, **d**, **g**, **h**). Strong immunoreactivity for α-synuclein and ubiquitin was observed in the somatodendritic compartment in the pons, deep cerebellar nucleus, and cervical cord in the homozygous mice. In the heterozygous mice, no or faint immunoreactivity was observed in the somatodendritic compartment (**d**, **h**). *Scale bar* 50 μm (**a–c**, **f**, **g**), 100 μm (**d**, **e**, **h**)

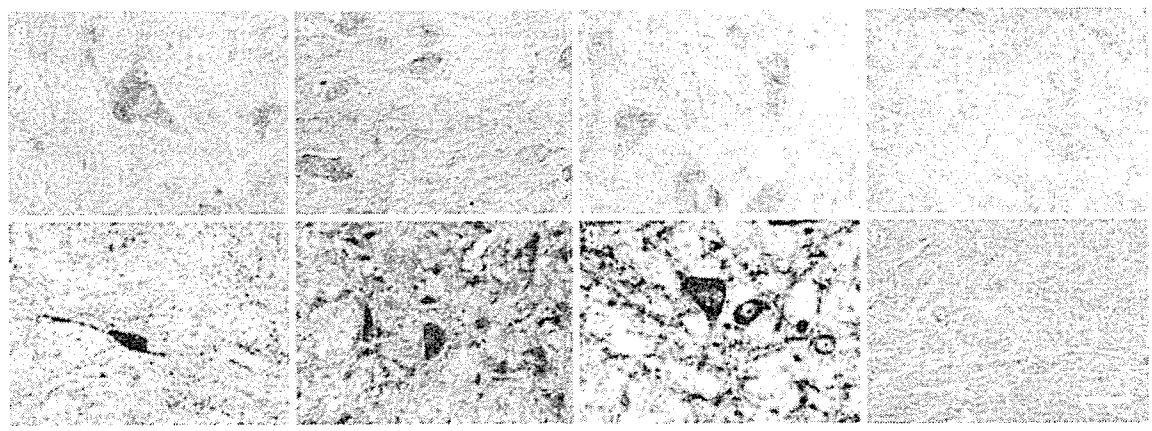

Fig. 2 Immunostaining for synphilin-1 (**a–d**) and 14-3-3 [**e–h**; **e** 14-3-3ζ, **f** 14-3-3β (A-15), **g–h** 14-3-3γ] in homozygous mice (**a–c**, **e–g**) and heterozygous mice (**d**, **h**). The photomicrographs were taken from the pons (**a**, **e**), deep cerebellar nucleus (**b**), lumbar spinal cord (**f**), and cervical spinal cord (**c**, **d**, **g**, **h**). Strong immunoreactivity for synphilin-1, 14-3-3ζ, 14-3-3β, and 14-3-3γ was observed in the somatodendritic compartment in the pons, deep cerebellar nucleus, and spinal cord in the homozygous mice. In the heterozygous mice, no or faint immunoreactivity was observed in the somatodendritic compartment (**d**, **h**). *Scale bar* 50 μm (**a–c**, **e–g**), 100 μm (**d**, **h**)

homozygous mice, strong 14-3-3β (A-15) immunoreactivity was observed in the granules of the neuronal somata and dendrites (Fig. 2f). Immunoreactivity for 14-3-3ε in the homozygous mice was localized to the neuronal nuclei in the cerebral cortex, brainstem, deep cerebellar nuclei, and spinal cord. In the heterozygous mice, 14-3-3ε immunoreactivity was observed also in the same areas as the homozygous mice, except for the spinal cord.

Immunoreactivity for 14-3-3η and σ in the homozygous mice was localized to the neuronal somatodendritic components in the cerebral cortex, brainstem, deep cerebellar nuclei, and spinal cord. Immunoreactivity for 14-3-3η and σ in the heterozygous mice was also observed in the same areas as the homozygous mice, and there were no remarkable differences in the distribution of 14-3-3η and σ between the homozygous and heterozygous mice.

Double immunofluorescent staining

In the homozygous mice, the distribution of immunoreactivity for synphilin-1 and 14-3-3 was mainly observed in the medial part of the midbrain and pons, DCN, and spinal cord, and was similar to that of α-synuclein (Fig. 4).

Double-immunofluorescent-stained sections in the homozygous mice showed that 14-3-3β and γ were colocalized with α-synuclein in the spinal cord. 14-3-3ζ was colocalized with α-synuclein in the pons. Synphilin-1 was also colocalized with α-synuclein in the pons (Fig. 3). By randomly sampling three arbitrary regions in the pons, the mean frequencies of double labeling for 14-3-3β, γ, θ, ζ, and ε immunoreactivities with α-synuclein were 83, 70, 66, 44, and 56%, respectively, in the homozygous mice.

Discussion

The most notable finding of this study using the A53T-Tg mice was that α-synuclein overexpression alone was enough to induce aggregates containing α-synuclein, 14-3-3 proteins, and synphilin-1, which are partially reminiscent of LBs. Given the fact that the difference between homozygous and heterozygous A53T-Tg mice is limited to the magnitude of expression of the transgene-derived human α-synuclein, and that the expres-

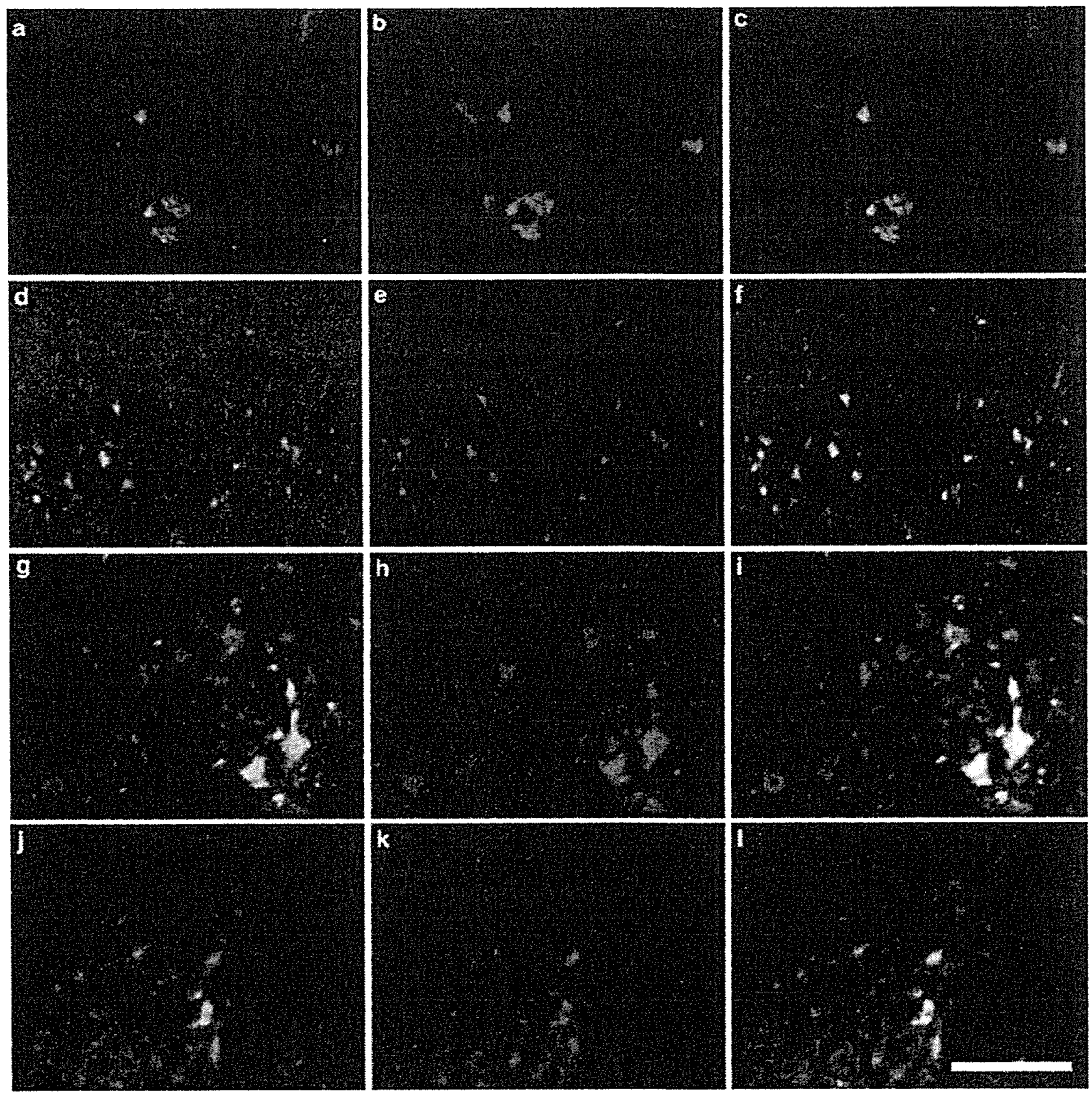

Fig. 3 Double immunofluorescent staining for α-synuclein plus synphilin-1 (**a–c**), and α-synuclein plus 14-3-3 (**d-l**) in homozygous mice. **a–f** pons; **g–i** lumbar spinal cord; and **j–l** thoracic spinal cord. The *left column* (**a**, **d**, **g**, **j**; *green*) shows immunostaining for α-synuclein, and the *middle column* shows (**b**, **e**, **h**, **k**; *red*) immunostaining for synphilin-1 (**b**), 14-3-3ζ (**e**), 14-3-3γ (**h**), and 14-3-3β (**k**). The *right column* (**c**, **f**, **i**, **l**) shows the merged image. *Scale bar* 50 μm (**a–c**), 100 μm (**d–l**)

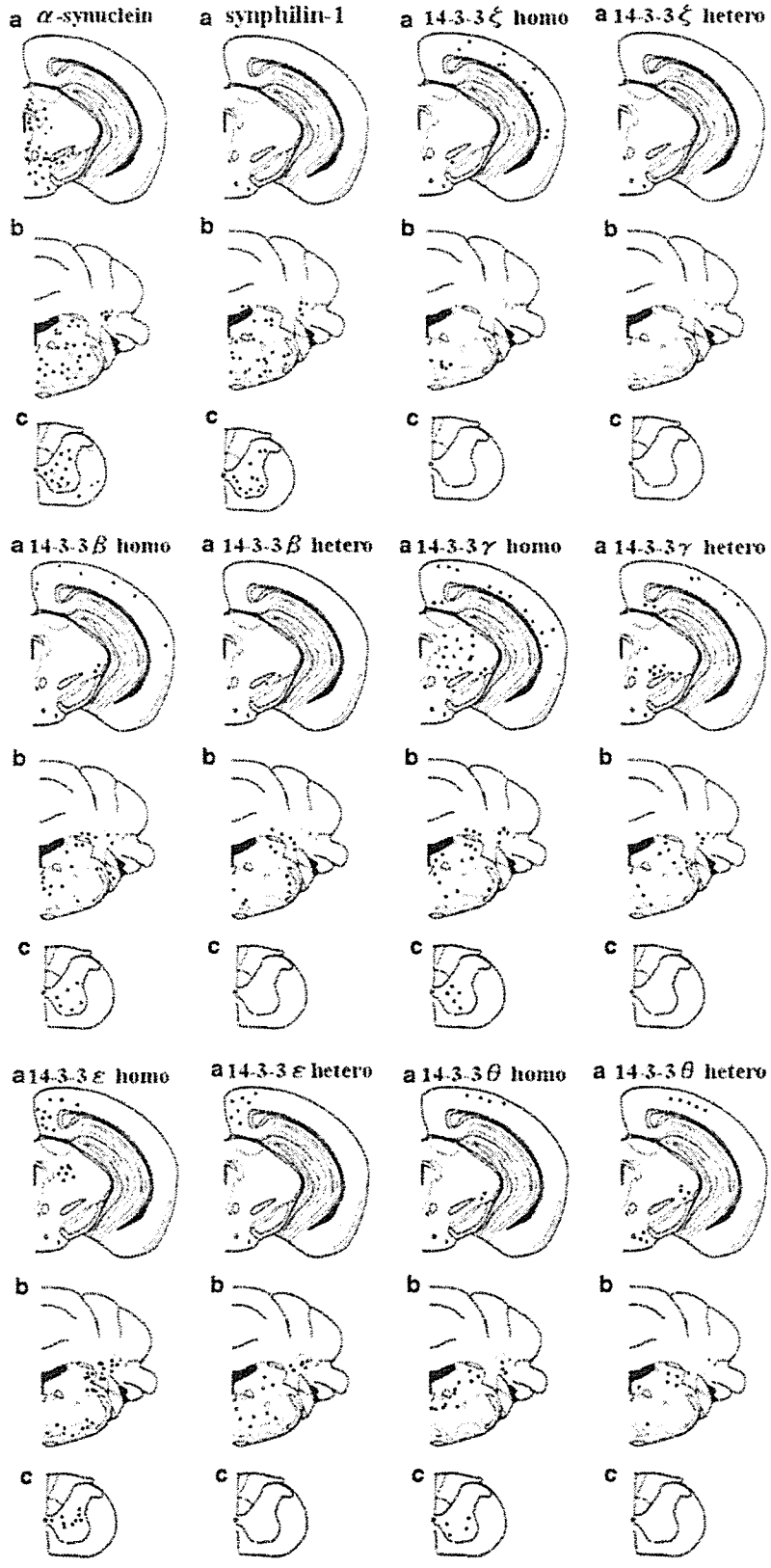

Fig. 4 Schematic distribution of α-synuclein (*α-syn*), synphilin-1 (*sph*), and 14-3-3 in A53T-Tg mice. A schematic summary of the α-synuclein, synphilin-1, and 14-3-3 pathology was shown as coronal sections from the A53T-Tg mouse brain and spinal cord, at the level of the midbrain (**a**), pons and deep cerebellar nucleus (**b**), and the cervical spinal cord (**c**). The *dots* represent one to three neurons with immunoreactivity for each antigen

sion level is approximately 20× higher than the endogenous expression observed in homozygous A53T-Tg mice [14], 14-3-3 proteins and synphilin-1 are more likely to be sequestered into the aggregates, rather than stoichiometrically associated with α-synuclein to form insoluble complexes. The sequestration of 14-3-3 proteins and synphilin-1 into aggregates may decrease the amount of functional 14-3-3 proteins and synphilin-1 available in the synaptic terminal, possibly disrupting chaperone and synaptic functions, respectively.

Under normal conditions, α-synuclein exists as a relatively unfolded molecule in the presynaptic terminals. However, under pathological conditions, it forms fibrils, accumulates in the neuronal somata, and may form inclusion bodies. In the heterozygous mice, α-synuclein has been reported to exhibit a neuropil immunostaining pattern [14], and this study showed a similar result without any immunoreactivities for α-synuclein in the somatodendritic compartment. Immunoreactivities for 14-3-3β and γ in the normal mouse brain have been localized to neurons in the brainstem and cerebral cortex, but not in the spinal cord [3]. These immunostaining patterns were similar in the heterozygous mice, in which the neuronal immunolabeling for 14-3-3β and γ occurred in the brainstem and cerebral cortex. In contrast, in the homozygous mice, 14-3-3γ accumulated in the spinal anterior horn cells (Fig. 4).

The immunostaining patterns for 14-3-3β in the homozygous mice have been strongly positive in the granules from the neuronal somata and dendrites in the spinal cord. The homozygous mice have been shown to develop motor impairments, possibly due to a degeneration of the anterior horn cells. Therefore, this accumulation of 14-3-3β and γ in the anterior horn cell bodies may play a significant role in the development of motor symptoms in these mice. Previously, we showed that LB-like hyaline inclusions in the anterior horn cells were immunoreactive for several 14-3-3 proteins in patients with amyotrophic lateral sclerosis [21]. Taken together, the accumulation of 14-3-3 proteins in the somatodendritic compartments of the anterior horn cells may be generally associated with a dysfunction of the lower motor neurons.

Synphilin-1A, an isoform of synphilin-1, is localized to the LBs and is an aggregation-prone protein that causes neuronal toxicity [11]. In this study, we used an anti-synphilin-1 antibody which recognizes the C-terminus of synphilin-1, and thus there was the possibility that this anti-synphilin-1 antibody may have cross-reacted, and showed the localization of synphilin-1A in the homozygous mice. Increasing evidence further supplements the conjugation of α-synuclein with 14-3-3 proteins or synphilin-1. In cell cultures, the co-transfection of synphilin-1 and α-synuclein has been shown to promote the formation of eosinophilic cytoplasmic inclusions like LBs [10]. Xu and colleagues [37] showed that soluble wild-type and mutant α-synuclein form complexes with 14-3-3 proteins, and accumulate in dopaminergic neurons. The colocalization of 14-3-3 proteins or synphilin-1 with α-synuclein in LBs has been demonstrated in human brains [22, 36]. Under non-pathological conditions, 14-3-3 proteins inhibit apoptosis by binding and inactivating pro-apoptotic proteins, including the mitochondrial Bcl-2 family member BAD and the transcription factor Forkhead [39]. Berg et al. [4] showed that 14-3-3 proteins, especially the ε, γ, θ, and ζ isoforms, were colocalized in the LBs. Kaneko and Hachiya [18] proposed the possibility that the distinctive function of 14-3-3ζ might be a sweeper of misfolded proteins such as aggregates or inclusion bodies. In our study, enhanced immunoreactivity for 14-3-3 proteins and synphilin-1 was observed in the A53T-Tg homozygous mice. According to these results, the 14-3-3 proteins bound to the overexpressed α-synuclein, and thus forming complexes of these proteins which may lead to the sequestration and subsequent decrease in 14-3-3 proteins. In turn, this decrease in 14-3-3 proteins causes the apoptotic neuronal cell death. Furthermore, those 14-3-3 proteins which interact with α-synuclein and accumulate in LBs may also interact with synphilin-1 [2]. The sequestration of synphilin-1 may also contribute to neuronal dysfunction, possibly through an impairment of synaptic function attributed to α-synuclein, although the exact role of synphilin-1 remains to be fully understood. The present study showed that α-synuclein colocalized with 14-3-3 proteins and synphilin-1, suggesting that these multi-protein complexes formed by α-synuclein, synphilin-1, and 14-3-3 proteins may account for the pathogenesis of neuronal cell death.

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A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin

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Abstract

It has been proposed that in autosomal recessive juvenile parkinsonism (AR-JP), a ubiquitin ligase (E3) Parkin, which is involved in endoplasmic reticulum-associated degradation (ERAD), lacks E3 activity. The resulting accumulation of Parkin-associated endothelin receptor-like receptor (Pael-R), a substrate of Parkin, leads to endoplasmic reticulum stress, causing neuronal death. We previously reported that human E3 HRD1 in the endoplasmic reticulum protects against endoplasmic reticulum stress-induced apoptosis. This study shows that HRD1 was expressed in substantia nigra pars compacta (SNC) dopaminergic neurons and interacted with Pael-R through the HRD1 proline-rich region, promoting the ubiquitylation and degradation of Pael-R. Furthermore, the disruption of endogenous HRD1 by small interfering RNA (siRNA) induced Pael-R accumulation and caspase-3 activation. We also found that ATF6 overexpression, which induced HRD1, accelerated and caused Pael-R degradation; the suppression of HRD1 expression by siRNA partially prevents this degradation. These results suggest that in addition to Parkin, HRD1 is also involved in the degradation of Pael-R.

Keywords: endoplasmic reticulum stress; endoplasmic reticulum-associated degradation; HRD1; Parkin-associated endothelin receptor-like receptor; Parkinson's disease; unfolded protein response

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Parkinson's disease is the most common movement disorder and the second most common neurodegenerative disease. Only approximately 5% of Parkinson's disease patients are familial. Autosomal recessive juvenile parkinsonism (AR-JP) occurs with increasing frequency in familial Parkinson's disease patients and results from parkin gene (*PARK2*) mutations (Kitada *et al.* 1998). In AR-JP patients, the loss of dopaminergic neurons and the appearance of parkinsonism symptoms occur without the formation of Lewy bodies,

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Abbreviations used: AR-JP, autosomal recessive juvenile parkinsonism; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ERAD, endoplasmic reticulum-associated degradation; E3, ubiquitin ligase; E2, ubiquitin-conjugating enzyme; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; Pael-R, Parkin-associated endothelin receptor-like receptor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; HRD, HMG-CoA reductase degradation; SEL, suppressor or enhancer of lin-12; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNC, substantia nigra pars compacta; UPR, unfolded protein response; XBP1, X-box binding protein 1; ATF6, activating transcription factor 6; UPS, ubiquitin-proteasome system.

which are a significant characteristic of non-familial and some familial Parkinson's disease cases (Mizuno *et al.* 1998).

Eukaryotic cells coordinate the folding and glycosylation of secretary and membrane proteins in the endoplasmic reticulum. Various stresses leading to impairment of the endoplasmic reticulum and the production of mutant proteins cause the accumulation of unfolded proteins in the endoplasmic reticulum, culminating in cell death. Unfolded proteins accumulated in the endoplasmic reticulum are degraded by the ubiquitin-proteasome system (UPS). In this endoplasmic reticulum system, termed endoplasmic reticulum-associated degradation (ERAD), unfolded proteins are initially retrotranslocated from the endoplasmic reticulum to the cytosol through the translocon, polyubiquitylated by ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and other components, and degraded by the 26S proteasome (Hershko and Ciechanover 1998). E3 plays an important role in the ubiquitylation of unfolded proteins, and the RING finger domain of E3 mediates the transfer of ubiquitin from E2 to substrates (Zheng *et al.* 2000).

Parkin is an E3 that contains two RING finger domains; AR-JP-linked Parkin mutants have defective E3 activity. Parkin is up-regulated in response to endoplasmic reticulum stress and protects against cell death caused by such stress, suggesting that it is an E3 involved in ERAD. Parkin-associated endothelin receptor-like receptor (Pael-R) has been identified as a protein that interacts with Parkin; its accumulation leads to endoplasmic reticulum stress-induced cell death. Parkin ubiquitinates and promotes the degradation of insoluble Pael-R, resulting in the suppression of cell death (Imai *et al.* 2001). In other words, endoplasmic reticulum stress caused by the accumulation of unfolded Pael-R might be involved in AR-JP. Furthermore, it has been recently reported that Pael-R in Parkinson's disease is accumulated in the core of Lewy bodies (Murakami *et al.* 2004) and that selective dopaminergic neurodegeneration is caused by the ectopic expression of human Pael-R in the *Drosophila* brain (Yang *et al.* 2003).

It is known that in yeast, Hrd1p/Der3p is involved in ERAD. Hrd1p/Der3p is localized in the endoplasmic reticulum, contains the RING-finger domain at the C-terminus, and ubiquitinates substrates including HMG-CoA reductase (Hmg2p) (Gardner *et al.* 2000, 2001; Deak and Wolf 2001). Hrd3p is reported to regulate or stabilize Hrd1p (Plemper *et al.* 1999; Deak and Wolf 2001). Endoplasmic reticulum stress induces various components involved in ERAD, including Hrd1p as well as endoplasmic reticulum molecular chaperones, suggesting that ERAD involves the degradation of unfolded proteins in cooperation with endoplasmic reticulum chaperones (Friedlander *et al.* 2000; Travers *et al.* 2000). We previously reported that human HRD1 was identified and characterized as a human homolog of yeast Hrd1p (Kaneko *et al.* 2002). In that report, we demonstrated that HRD1 possesses E3 activity, is induced during endoplasmic reticulum stress, and suppresses cell death caused by endoplasmic reticulum stress. Furthermore, human HRD1 is reportedly involved in the basal, and not the sterol-regulated, degradation of HMG-CoA reductase (Nadav *et al.* 2003; Kikkert *et al.* 2004) and is a pathogenic factor in rheumatoid arthritis (Amano *et al.* 2003).

The unfolded protein response (UPR) is required for the inhibition of further protein synthesis and the induction of endoplasmic reticulum chaperones, which reduce the number of unfolded proteins in the endoplasmic reticulum (Kaufman 1999, 2002). Transcription factor ATF6 is a transmembrane protein localized in the endoplasmic reticulum (Haze *et al.* 1999). Under endoplasmic reticulum stress, ATF6 is cleaved to release the N-terminal fragment on the cytosolic side of the membrane; it then enters the nucleus, acts as a transcription factor, and eventually activates endoplasmic reticulum chaperone gene transcription, which enhances protein folding (Haze *et al.* 1999; Ye *et al.* 2000; Shen *et al.* 2002). On the other hand, an endoplasmic reticulum-resident transmembrane protein IRE1, which possesses serine/threonine kinase and RNase domains, is dimerized and autophosphorylated during endoplasmic reticulum stress (Cox *et al.* 1993; Sidrauski and Walter 1997). Activated IRE1 splices XBP1 mRNA and then generates an active form of XBP1 (Yoshida *et al.* 2001).

Recent studies have demonstrated that Parkin knockout mice exhibit no significant change in either dopaminergic neurodegeneration or the accumulation of any Parkin substrates (Goldberg *et al.* 2003; Itier *et al.* 2003; Palacino *et al.* 2004; Von Coelln *et al.* 2004; Perez and Palmiter 2005; Periquet *et al.* 2005), suggesting that other unknown E3s can degrade accumulated proteins in the absence of Parkin. On the other hand, HRD1 apparently degrades a number of unfolded proteins as overexpressed HRD1 protects against endoplasmic reticulum stress-induced cell death. This study showed that human HRD1 was located in substantia nigra pars compacta (SNC) neurons in the mouse brain. Therefore, we hypothesized that HRD1 as well as Parkin ubiquitinates and degrades the unfolded Pael-R responsible for endoplasmic reticulum stress and protects against Pael-R-induced cell death. In addition, we investigated whether ATF6-induced UPR activation promotes the degradation of Pael-R and whether UPR-induced HRD1 expression is partially involved in this degradation.

Materials and methods

Constructs

The expression vector for human wild-type and truncated fragments of HRD1 was tagged with myc and polyhistidine (6 × His) epitopes at the C-terminus of the inserted sequence (pcDNA6; Invitrogen Corporation, Carlsbad, CA, USA). Human Pael-R (pcDNA3),

tagged with FLAG and 6 × His epitopes at the C-terminus, was a gift from Ryosuke Takahashi (RIKEN Brain Science Institute, Japan). The expression vector for wild-type human α-synuclein, tagged with hemagglutinin and 6 × His epitopes at the C-terminus, was cloned into expression vector pcDNA3.1 (Invitrogen). The expression vector for the cleaved form of ATF6 (amino acid region 1–373 of ATF6α), tagged with hemagglutinin epitopes at the N-terminus was cloned into expression vector pCR3.1 (Invitrogen). The expression vector for RP-HRD1 fused at its N-terminus to glutathione S-transferase (GST) was cloned into the expression vector pGEX6p-1 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Affinity-purified antibodies, chemicals, and proteins

HRD1 polyclonal antibody against the KLH-conjugated synthetic peptide (C)-EDGEPDAAELRRR, corresponding to amino acid residues 594–606 of human HRD1 protein, was recognized human and mouse HRD1 (a gift from Otsuka GEN Research Institute). We also purchased anti-HRD1 polyclonal antibody (C-term) from Abgent (San Diego, CA, USA). Anti-Pael-R polyclonal antibody was used as described (Imai *et al.* 2001). Anti-FLAG M2 polyclonal and HRP-conjugated M2 monoclonal antibodies, and M2 affinity gel were purchased from Sigma-Aldrich (St. Louis, MO, USA); anti-calreticulin polyclonal and anti-KDEL monoclonal antibodies were from Stressgen Biotechnologies Corporation (Ann Arbor, MI, USA); anti-myc monoclonal (9E10) antibody was from Oncogene Research Products (Cambridge, MA, USA); anti-caspase-3 (Asp175) polyclonal antibody was from Cell Signaling Technology Inc. (Danvers, MA, USA); anti-GST polyclonal (Z-5) and anti-hemagglutinin polyclonal (Y-11) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); MG132 was from the Peptide Institute (Osaka, Japan), and rabbit ubiquitin-activating enzyme (E1), GST-UbcH5c (E2), and GST-ubiquitin were from BostonBiochem (Cambridge, MA, USA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (both from GE Healthcare Bio-sciences) were used as the secondary antibody. Bands were detected using the enhanced chemiluminescence (ECL) system (GE Healthcare Bio-Sciences).

Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde, processed on a Tissue-Tek VIP (Sakura Finetek, Tokyo, Japan), and then embedded in paraffin. The brains were sectioned into 4-μm-thick slices, mounted on silane-coated slides, and then subjected to heat treatment with 10 mM sodium citrate buffer (pH 6.0) in a pressure cooker for 3 min. Diaminobenzidine (DAB) immunostaining was performed using anti-HRD1 polyclonal antibody as the primary antibody (1 : 50 dilution), a peroxidase-labeled polymer-conjugated anti-rabbit antibody (Envision system; Dako, Glostrup, Denmark), and DAB as the substrate.

Immunofluorescence staining was stained with anti-HRD1 polyclonal antibody (1 : 20 dilution) and either anti-neuron-specific nuclear protein (NeuN; 1 : 100 dilution; Chemicon International, Temecula, CA, USA), anti-glial fibrillary acidic protein (GFAP; 1 : 100 dilution; Chemicon International), or anti-tyrosine hydroxylase (1 : 100 dilution; Chemicon International) monoclonal antibodies, and then with anti-mouse antibody conjugated with Alexa 546 and anti-rabbit antibody with Alexa 488 (Molecular Probes, Eugene, OR, USA). Fluorescence images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Gottingen, Germany).

Immunocytochemistry

For the subcellular localization of HRD1 and Pael-R, COS-1 cells were transfected with HRD1-myc or a control vector (Mock) and Pael-R-FLAG using the calcium phosphate method. To visualize the effect of HRD1 degrading Pael-R, normal human embryonic kidney (HEK293) cells and those stably transfected with HRD1-myc and M-HRD1-myc were transfected with Pael-R-FLAG-pcDNA3 and DsRED-express-N1 vector (Promega, Madison, WI, USA) using LipofectAMINE 2000 (Invitrogen). At 36 h after transfection, the cells were fixed with methanol at −20°C. The cells were then stained for the presence of proteins with appropriate primary antibodies, and then with anti-mouse antibody conjugated with Alexa 488 and/or anti-rabbit antibody with Alexa 594 (Molecular Probes). Fluorescence images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Gottingen, Germany).

Immunoprecipitation and western blotting

Transfected HEK293 cells were lysed in a lysis buffer [20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% Triton X-100 with complete protease inhibitors (Roche Diagnostics K.K., Basel, Switzerland)]. Immunoprecipitation was carried out by incubating the supernatant with the indicated antibodies for 16 h and then with Protein G Sepharose Fast Flow (GE Healthcare Bio-sciences) for 1 h. For immunoprecipitation with an anti-FLAG antibody, the supernatant was incubated with anti-FLAG M2 affinity gel for 16 h. The immune complex was rinsed with a washing buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, and 0.2% Triton X-100].

Pulse-chase experiment

Neuro2a cells were transfected with Pael-R-FLAG and either a control vector, HRD1-myc or M-HRD1-myc. At 36 h after transfection, the cells were starved for 1 h in methionine/cysteine-free Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 5% dialyzed fetal calf serum (FCS), and then labeled for 1 h at 37°C with 100 μCi/mL [^{35}S]-methionine/cysteine (Redivue Promix L-[^{35}S] *in vitro* cell labeling mix; GE Healthcare Bio-Sciences). The cells were then washed and incubated in DMEM containing 10% FCS for the indicated periods. The cell lysates were immunoprecipitated with the anti-FLAG antibody, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and visualized using an imaging analyzer (BAS-2500, Fujifilm, Tokyo, Japan). The metabolically labeled Pael-R was quantified using Image Gauge software (Fujifilm).

Cell death assay

Normal HEK293 cells and those stably expressing HRD1-myc or M-HRD1-myc were transfected with a control vector or Pael-R-FLAG and incubated for 24 h. The cells were washed with phosphate-buffered saline (PBS) and then stained with crystal violet (0.1% crystal violet, WAKO Pure Chemical Industries, Osaka, Japan), and the wells washed with water and air-dried. The dye was eluted with water containing 0.5% SDS, and optical density was measured at 590 nm.

In vitro ubiquitylation assay

RING-proline (RP)-HRD1-myc and Pael-R-FLAG were produced by T_NT quick-coupled transcription/translation systems (Promega). Sixteen microliters of T_NT reaction lysates producing RP-HRD1 or Pael-R-FLAG were mixed with other components including E1 (25 ng), E2 (GST-UbcH5c, 400 ng), and GST-ubiquitin (7 ng) in 100 μL of reaction buffer [40 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, and 2 mM dithiothreitol (DTT)]. The reaction mixtures were incubated at 30°C for 90 min, immunoprecipitated with anti-FLAG antibody, subjected to SDS-PAGE, and analyzed by western blotting using the anti-GST polyclonal antibody.

In vitro binding assay

RP-HRD1 was cloned into the pGEX 6p-1 vector (GE Healthcare Bio-Sciences). GST-RP-HRD1 and GST were expressed by culturing *Escherichia coli* DH5α with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The cells were collected and lysed in a lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10%, 0.5% Triton X-100 with 1.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The supernatants were mixed with glutathione-Sepharose 4B (GE Healthcare Bio-Sciences) for 16 h at 4°C. The beads were washed with lysis buffer and eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione, and the eluted fraction dialyzed against PBS.

Equal amounts of purified GST or GST-RP-HRD1 were applied to glutathione Sepharose 4B in a binding buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.25% gelatin, and 1% Triton X-100 at 4°C for 16 h, and then washed with the buffer. T_NT reaction lysates producing ^{35}S-labeled Pael-R-FLAG were incubated with aliquots of GST- or GST-RP-HRD1-coupled glutathione-Sepharose 4B for 2 h at 4°C in the binding buffer. After extensive washing of the column with a washing buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100, the proteins recovered from the resin were subjected to SDS-PAGE followed by Coomassie blue staining and then visualized using an imaging analyzer (BAS-2500, Fujifilm).

RNA interference

For HRD1 knockdown by RNA interference, siGENOME SMARTpools of four oligoduplexes targeted against HRD1 (M-007090–00; Dharmacon Research, Lafayette, CO, USA) were used. Small interfering RNA (siRNA) transfection was performed using 100 pmol of siRNA and 7.5 μL of LipofectAMINE 2000 reagent (Invitrogen) in 6 cm dishes.

Results

Localization of HRD1 and Pael-R in the murine brain and cellular endoplasmic reticulum

As HRD1 has been shown to be highly expressed in the human fetal brain by RT-PCR-ELISA (Nagase *et al.* 2001), we immunohistochemically examined where HRD1 is localized in the murine brain. DAB staining showed HRD1 expression was observed in SNC neurons, which are selectively degenerated in Parkinson's disease (Fig. 1a), as well as in pyramidal cells of the hippocampus and Purkinje cells of the cerebellum (data not shown). Fluorescence staining using anti-NeuN and anti-GFAP antibodies showed that HRD1 was widely expressed in neuronal cells but not in glial cells (Fig. 1b). Furthermore, HRD1-immunoreactive cells were partially tyrosine hydroxylase-positive, indicating that HRD1 was expressed in dopaminergic neurons in the SNC (Fig. 1c). Thus, we hypothesized that HRD1 exists in the substantia nigra together with Pael-R as Pael-R is expressed in SNC dopaminergic neurons (Imai *et al.* 2001). To examine the subcellular localizations of HRD1 and Pael-R, expression vectors for HRD1-myc or the control vector (Mock) and Pael-R-FLAG were transfected into COS-1 cells. The localization of HRD1 (*green*) almost completely overlapped that of endogenous calreticulin (*red*) as revealed by an endoplasmic reticulum marker (Fig. 1d, lower). Pael-R (*red*) was widely localized in the endoplasmic reticulum as well as the cell surface and partially colocalized with HRD1 (*green*) in the endoplasmic reticulum (Fig. 1d, upper). Furthermore, endogenous HRD1 (*green*) was partially colocalized with Pael-R (*red*) in Pael-R-FLAG-expressing SH-SY5Y cells (Fig. 1e).

HRD1 interacts with unfolded Pael-R

When Pael-R was overexpressed in HEK293 cells, Pael-R proteins migrated as high molecular mass broad smears (Fig. 2a, lane 2), suggesting that they had undergone covalent modifications (glycosylation, ubiquitylation, etc.) (Imai *et al.* 2001); however, in the transfection of Pael-R with hemagglutinin-Ub, the ubiquitylation of Pael-R was barely observed in the absence of proteasome inhibitor MG132 (Fig. 2a, lane 5). Therefore, we presumed that the high molecular mass broad smears observed were the result of the aggregate formation of detergent-insoluble Pael-R rather than ubiquitylated Pael-R. Next, we used the immunoprecipitation method to investigate whether HRD1 interacts with Pael-R. HRD1 protein was detected in anti-FLAG antibody immunoprecipitates from cells cotransfected with HRD1-myc and Pael-R-FLAG (Fig. 2b, lane 15). In addition, Pael-R protein was detected in immunoprecipitates with an anti-myc antibody (Fig. 2b, lane 3), indicating that HRD1 interacts with Pael-R.

Furthermore, we performed coimmunoprecipitation in SH-SY5Y cells that stably expressed Pael-R-FLAG. The endogenous HRD1 protein was detected in immunoprecipitates with overexpressed aggregated Pael-R (Fig. 2c, upper and lower, lane 4). To investigate the interaction between HRD1 and Pael-R under a wider range of physiological conditions, the endogenous proteins in dopaminergic neuroblastoma SH-SY5Y cells were coimmunoprecipitated with the anti-Pael-R antibody; however, HRD1 was not coimmunoprecipitated with Pael-R (Fig. 2d, lane 3) under normal conditions. As Pael-R is easily unfolded and becomes insoluble under endoplasmic reticulum stress, we investigated the interaction