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Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain

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Parkin, a product of the causative gene of autosomal-recessive juvenile parkinsonism (AR-JP), is a RING-type E3 ubiquitin ligase and has an amino-terminal ubiquitin-like (Ubl) domain. Although a single mutation that causes an Arg to Pro substitution at position 42 of the Ubl domain (the Arg 42 mutation) has been identified in AR-JP patients, the function of this domain is not clear. In this study, we determined the three-dimensional structure of the Ubl domain of parkin by NMR, in particular by extensive use of backbone ^{15}N-^{1}H residual dipolar-coupling data. Inspection of chemical-shift-perturbation data showed that the parkin Ubl domain binds the Rpn10 subunit of 26S proteasomes via the region of parkin that includes position 42. Our findings suggest that the Arg 42 mutation induces a conformational change in the Rpn10-binding site of Ubl, resulting in impaired proteasomal binding of parkin, which could be the cause of AR-JP.

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INTRODUCTION

Autosomal-recessive juvenile parkinsonism (AR-JP), one of the most common forms of familial Parkinson's disease, is characterized by selective and massive loss of dopaminergic neurons in the substantia nigra of the midbrain and by the absence of Lewy bodies (cytoplasmic inclusion bodies), which consist of aggregates of abnormally accumulated proteins (Yamamura *et al.*, 1973). The causative gene of AR-JP, *parkin*, encodes a 52-kDa protein composed of three parts: the amino-terminal ubiquitin (Ub)-like domain (Ubl), the carboxy-terminal RING-finger box and the linker region, which connects the two domains (Kitada *et al.*, 1998). Recently, parkin was shown to be a RING-type ubiquitin ligase (or E3 protein) that catalyses protein ubiquitylation, leading to proteasome-mediated protein degradation (Imai *et al.*, 2000; Shimura *et al.*, 2000; Zhang *et al.*, 2000). Analysis of *parkin* mutations in AR-JP patients has revealed that the molecular basis of this disease is the loss of parkin E3-enzyme function in the ubiquitin–proteasome pathway, which may result in the accumulation of parkin substrates in neurons (Chung *et al.*, 2001; Imai *et al.*, 2001; Shimura *et al.*, 2001).

The C-terminal RING box serves as a recruiting motif for ubiquitin-conjugating enzymes (E2 enzymes), such as Ubc4, Ubc7, UbcH7 and UbcH8, whereas the functional role of the N-terminal Ubl domain is poorly characterized. The number of identified mutations in the *parkin* gene in patients with early-onset parkinsonism has recently increased (Lücking *et al.*, 2000), and a single mutation that causes an Arg to Pro substitution at amino-acid position 42 of the Ubl domain has been identified in one family of AR-JP patients (Terreni *et al.*, 2001). This mutated parkin protein retains the ability to bind UbcH7, but fails to co-immunoprecipitate ubiquitylated proteins such as *O*-glycosylated α-synuclein (Shimura *et al.*, 2001). These data suggest that the Ubl domain contributes to the recognition of target proteins.

However, accumulating evidence indicates that various proteins with Ubl domains, such as Rad23, Dsk2 and their human homologues (HR23a/b and PLIC1/2, respectively), provide links between 26S proteasomes and the ubiquitylation machinery (Hiyama *et al.*, 1999; Kleijnen *et al.*, 2000; Wilkinson *et al.*, 2001). All of these proteins have Ubl domains at their N termini, and ubiquitin-associated (UBA) domains at their C termini; the Ubl domains are able to interact

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with 26S proteasomes, whereas the UBA domains can bind to polyubiquitin chains (Buchberger, 2002). However, it has been reported that a 50-kDa subunit of human 26S proteasomes, originally called S5a, can bind to polyubiquitin conjugates *in vitro*, and could therefore possibly function as a polyubiquitin-binding subunit (Deveraux *et al*., 1994). Several S5a homologues (such as Mbp1, Mcb1, Sun1, Pus1, μ-54 and Rpn1) have been identified in various eukaryotes (Kawahara *et al*., 2000). Here, we call S5a and its homologues 'Rpn10' on the basis of a recent proposal (Finley *et al*., 1998). Intriguingly, Rpn10 also binds the Ubl domains of HR23a/b and PLIC2 (Hiyama *et al*., 1999; Walters *et al*., 2002). Mutagenesis studies have shown that Rpn10 has two highly conserved polyubiquitin-binding sites, PUbS1 and PUbS2, each of which contains five hydrophobic residues forming an alternating pattern of large and small side-chains (Young *et al*., 1998). In addition, a homologous motif with a similar structure, known as a ubiquitin-interacting motif (UIM), which physically interacts with ubiquitin and/or polyubiquitylated chains, was recently identified in various proteins (Hofmann & Falquet, 2001). NMR studies have shown that the Ubl domains of HR23a and PLIC2 share a highly conserved hydrophobic surface for binding Rpn10 (Walters *et al*., 2002). In the Ubl domain of parkin, some of the residues corresponding to those in the hydrophobic Rpn10-binding surface are substituted with polar residues. Therefore, one cannot predict whether parkin can bind 26S proteasomes through interaction with the Rpn10 subunit.

In this study, we have determined the three-dimensional (3D) structure of the Ubl domain of parkin by NMR spectroscopy. Based on chemical-shift-perturbation data, we provide evidence that Ubl interacts with Rpn10 using a surface almost identical to the Rpn10-binding surfaces of the Ubl domains of HR23a and PLIC2. The Rpn10-binding site determined in this study includes position 42 of the parkin Ubl domain. We also discuss how these results relate to the molecular cause of AR-JP.

Table 1 | Statistics for NMR structure calculations

Overhauser-effect distance restraints	
Total number	489
Inter-residue	159
Medium range	71
Long range	66
Intra-residue	193
Number of hydrogen bonds	20
Number of residual dipolar-coupling restraints	129
Dihedral-angle restraints	
ϕ	41
ψ	41
Mean r.m.s. deviation of backbone atoms from the average structure (Å)	0.371 ± 0.117
Mean r.m.s. deviation of all heavy atoms from the average structure (Å)	1.025 ± 0.086
Deviation from idealized covalent geometry	
Bonds (Å)	0.005 ± 0.000
Angles (°)	0.684 ± 0.012
Impropers (°)	0.697 ± 0.012
Ramachandran plot (%)	
Residues in most favourable region	69.1
Residues in additionally allowed region	26.5
Residues in generously allowed region	4.4
Residues in disallowed region	0

RESULTS AND DISCUSSION

The solution structure of the parkin Ubl domain

The Ubl domain of parkin, comprising residues 1–76 of the full-length protein, was produced as a recombinant protein in *Escherichia coli*. Preliminary NMR studies revealed that the Ubl domain forms a disulphide-linked dimer, causing a large amount of aggregation during spectral measurement. Therefore, the solution conditions were optimized by adding 10 mM [$^2H_{10}$] dithiothreitol (DTT). However, the concentration of Ubl never exceeded 0.1 mM, even under these reducing conditions. Therefore, we carried out all spectral measurements using the parkin Ubl domain at a concentration of 0.1 mM. Gel-filtration analysis showed that the Ubl domain was monomeric in the solution conditions used (data not shown). Because extensive collection of the inter-proton restraints based on the nuclear Overhauser enhancement spectroscopy (NOESY) spectra was difficult to carry out, due to dilution of the sample solution (approximately 5–10% of the concentration typically used), the structure determination relied heavily on orientational restraints with respect to the NMR magnetic field. We chose two orientational media: bicelle and cetyltriammonium bromide (CTAB)-doped bicelle (see Methods) for measuring the backbone ^{15}N-^1H residual dipolar couplings (RDCs). The NMR data used for structure calculations are summarized in Table 1. A final set of ten structures was selected for 50 restrained molecular dynamics calculations for the Ubl domain, based on agreement with the experimental data and overall structural quality according to the following prerequisites: no nuclear Overhauser effect (NOE) violations >0.5 Å, no torsion angle violation >5° and no RDC violations >2 Hz (over a 0.6 Hz range) (Fig. 1A). The secondary structure of the parkin Ubl domain consists of two α-helices (α1: residues Ile 23–Arg 33; α2: Gln 57–Asp 60) and five β-sheets (β1: Ile 2–Phe 7; β2: His 11–Val 15; β3: Arg 42–Phe 45; β4: Lys 48–Glu 49; β5: Gln 64–Val 70). These are arranged in a typical ubiquitin fold (Fig. 1B and C). The average r.m.s. deviations from the average structure within the secondary structural elements for backbone atoms and for all heavy atoms are 0.371 Å and 1.025 Å, respectively.

Interaction of the parkin Ubl domain with Rpn10

To analyse the interaction of Rpn10 with the Ubl domain of parkin, we used the Rpn10 fragment comprising residues 196–306 ($Rpn10_{196–306}$), which retains both PUbS1 and PUbS2. Figure 2A shows a comparison of the ^1H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra of the uniformly-[^{15}N]-labelled Ubl domain in the presence and absence of $Rpn10_{196–306}$. Twenty-one significantly perturbed amide resonances were observed, whereas the rest of the spectrum remained unchanged, indicating the formation of a specific complex between Ubl and $Rpn10_{196–306}$ (Fig. 2B). The perturbed residues were mapped onto the Ubl domain structure (Fig. 2C). The identified contact-surface comprised the β3 and β4 strands of the parkin Ubl domain and the residues in their spatial proximity, which corresponds with the Rpn10-binding surfaces of the Ubl domains of PLIC2 and HR23a (Walters *et al*., 2002).

The Rpn10-binding sites of PLIC2 and HR23a have been characterized by the surface clusters of the hydrophobic residues that are bound by basic residues on the molecular surface, whereas the ubiquitin-binding site of Rpn10 seems to be a hydrophobic area bound by acidic residues (Walters *et al*., 2002). In PLIC2, the hydrophobic cluster comprises residues Ile 75, Ala 77, Ile 80, Val 101 and Ile 102, which correspond to Ile 44, Ala 46, Glu 49, Val 70 and Gln 71, respectively, in parkin. Mutagenesis studies have shown that hydrophobicity at

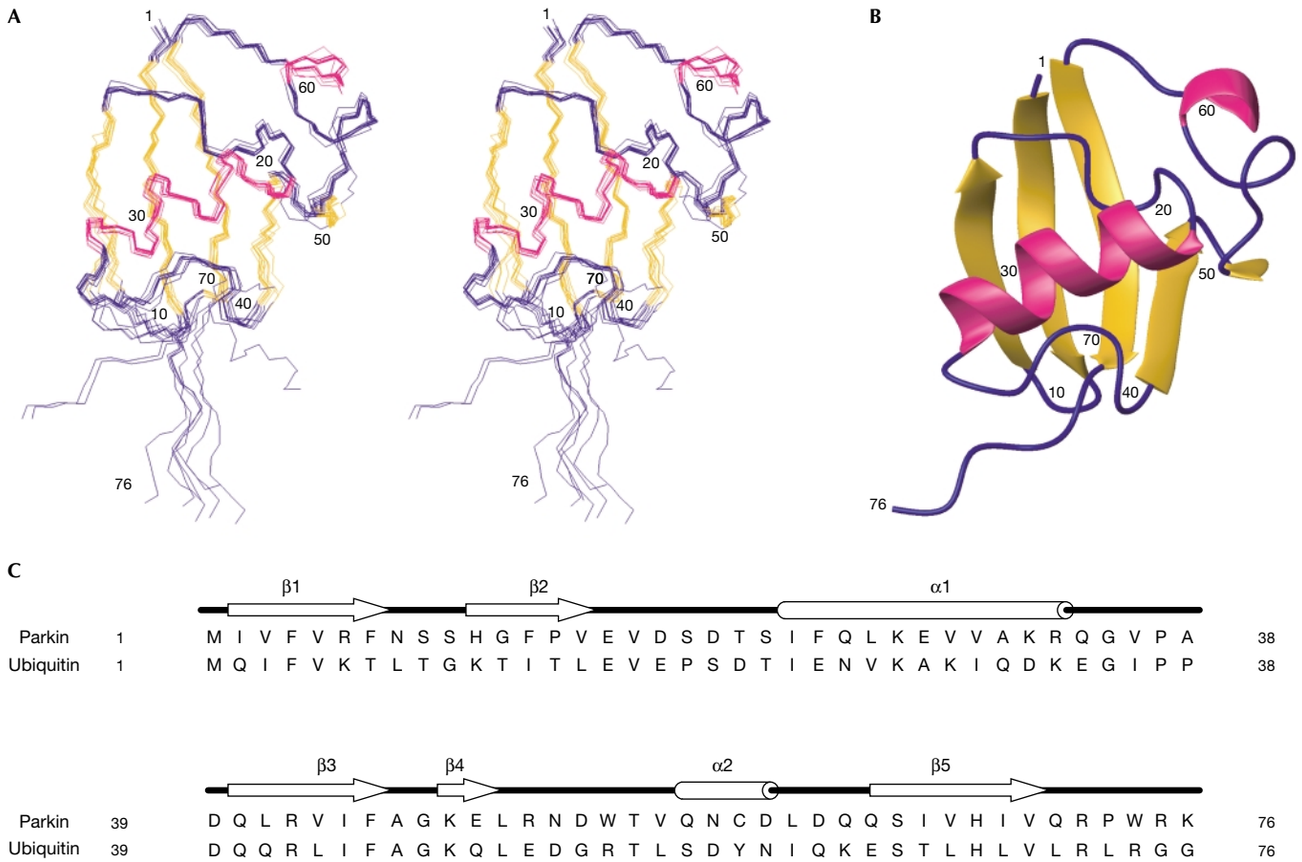

Fig. 1 | The solution structure of the ubiquitin-like (Ubl) domain of parkin. (**A**) Stereo view of ten converged structures of the parkin Ubl domain. (**B**) Ribbon representation of the average structure. β-strands and α-helices are coloured yellow and pink, respectively, in (**A**) and (**B**). Numbers in (**A**) and (**B**) indicate amino-acid positions in the Ubl domain sequence. (**C**) Sequence alignment and secondary-structure elements of the parkin Ubl domain and ubiquitin.

amino-acid position 8 in tetraubiquitin is required for its binding to Rpn10 (Beal *et al*., 1998). Position 8 is occupied by Leu, Pro and Asn in ubiquitin, PLIC2 and parkin, respectively. It should be noted that parkin also has the ability to bind Rpn10, using the surface area corresponding to the hydrophobic cluster of PLIC2 and HR23a, notwithstanding the fact that half of the positions in this cluster are occupied by polar residues in parkin. On the basis of the NMR data from this study, we suggest that interactions between the Rpn10 and Ubl domains are not soley due to the hydrophobic properties of the Ubl domains. It is possible that the relative contributions of the two ubiquitin-binding sites, PUbS1 and PUbS2, are different when binding to different Ubl domains. Indeed, little or no chemical-shift-perturbation was observed for the Ubl domain of parkin after addition of PUbS1 or PUbS2 alone (data not shown). This is in contrast with the finding that the PUbS2 region can interact with the Ubl domain of PLIC2 and HR23a (Walters *et al.*, 2002), suggesting that the modes of the interactions between the Rpn10 and Ubl domains are different, depending on the particular Ubl-domain-containing protein.

Several lines of evidence suggest that E3 proteins associate with 26S proteasomes, thereby recruiting the ubiquitylation machinery (Xie & Varshavsky, 2000; Jäger *et al*., 2001). The NMR data presented here show the structural basis for the proteasomal binding of E3. Recent studies suggest that the ATPase subunit of 26S proteasomes, not the Rpn10 subunit, is responsible for binding to the polyubiquitin chain (Lam *et al*., 2002). Taking the data in the present study into consideration, we suggest that parkin and a polyubiquitin-tagged substrate come together on 26S proteasomes to form an efficient assembly line for protein degradation.

Our preliminary experiments revealed that a region of endogenous parkin was co-immunoprecipitated with 26S proteasomes in extracts from Nero2a cells and from mouse brain extracts (data not shown), suggesting that parkin interacts with 26S proteasomes, presumably through the Rpn10 subunit. However, detection of a physical interaction between a glutathione-*S*-transferease (GST)-fused Ubl domain of parkin and FLAG-tagged Rpn10 by co-immunoprecipitation/western blot analysis was unsuccessful, although a clear interaction between HR23a and Rpn10 was seen under the same conditions (see supplementary information online), indicating that the parkin–Rpn10 interaction is weak. Alternatively, it is possible that tagging of parkin and Rpn10 may prevent any interaction between them under *in vitro* conditions. However, in yeast, other proteasome subunits—Rpn1 and/or Rpn2—also bind the ubiquitin-like domain of Rad23 (the yeast

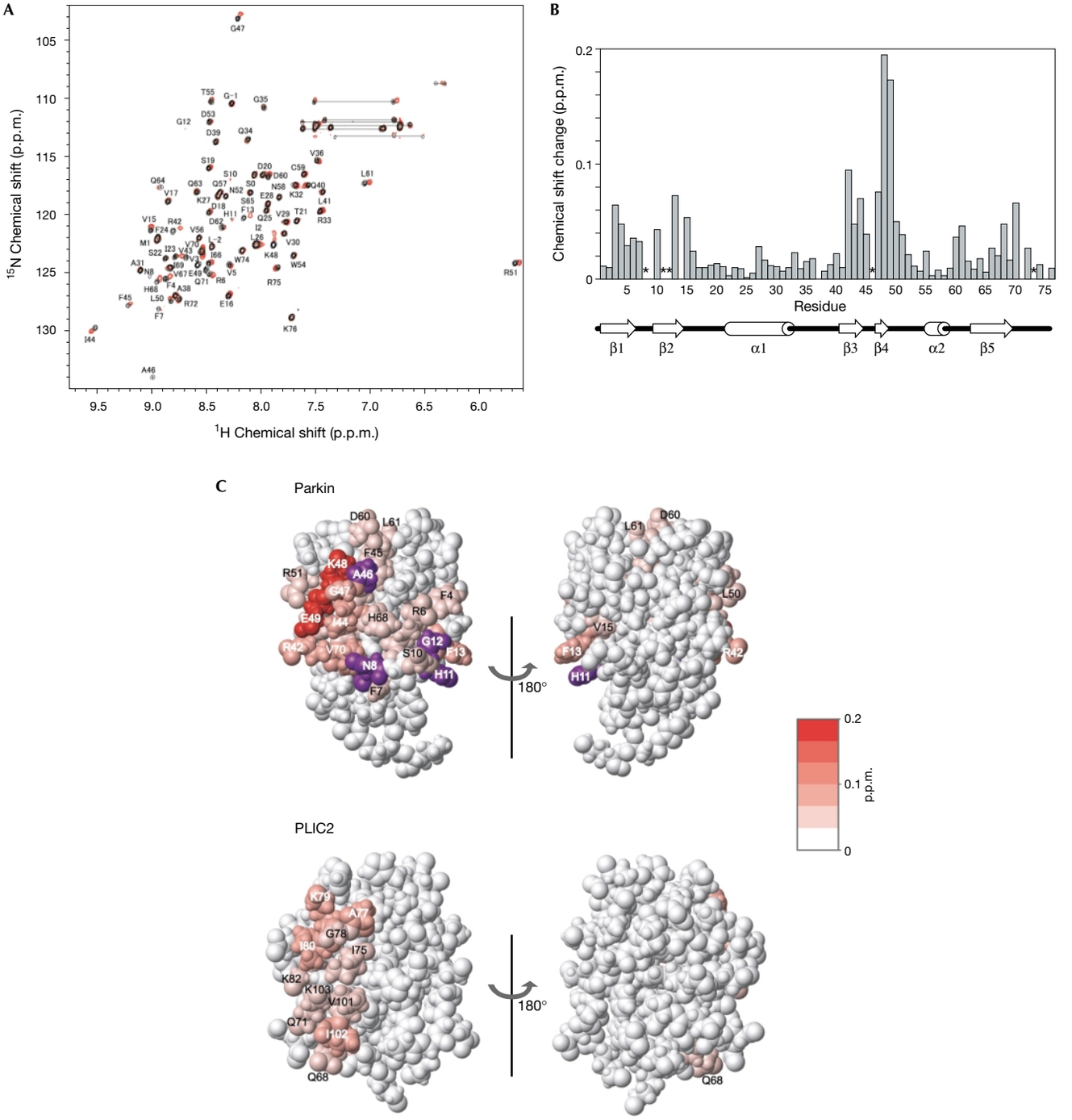

Fig. 2 | Identification of the binding site for $Rpn10_{196–306}$ in the parkin ubiquitin-like (Ubl) domain. (**A**) ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectrum of the parkin Ubl domain in the presence (red) and absence (black) of equimolar quantities of $Rpn10_{196–306}$. The peaks labelled with L-2, G-1 and S0 originate from the amino-terminal tag. (**B**) NMR chemical-shift-perturbation data for the parkin Ubl domain. The data are displayed for each residue according to the equation $(0.2\,\delta_N^2 + \delta_H^2)^{1/2}$, where δ_N and δ_H represent the change in nitrogen and proton chemical shifts on addition of $Rpn10_{196–306}$. Asterisks indicate residues the peaks of which became undetectable due to broadening. Secondary structure elements for the parkin Ubl are shown below the graph. (**C**) Mapping of the perturbed residues of the Ubl domains of parkin and PLIC2 (Walters *et al.*, 2002) on binding to Rpn10. Residues showing a chemical-shift-perturbation are coloured in red, with the colour gradient indicating the strength of the perturbation. Residues the peaks of which became undetectable on binding to Rpn10 are shown in purple.

homologue of HR23a), when only Rpn1 and/or Rpn2 are assembled into the 26S proteasome complex (Elsasser *et al*., 2002; Saeki *et al*., 2002). Therefore, the possibility of an interaction of parkin with Rpn1 and/or Rpn2, as well as with Rpn10, cannot be excluded. It is of note that yeast Rpn10 lacks the PUbS2 sequence (Kawahara *et al*., 2000) and that the human PUbS2 region, but not the PUbS1 region, can only interact with HR23a (Hiyama *et al*., 1999). However, it is unclear whether the 26S proteasome subunit(s) that functions as a Ubl-domain acceptor has changed during evolution. The determination of whether both Rpn1/Rpn2 and Rpn10 function redundantly as Ubl-domain acceptors, or whether they have distinct functions, awaits further studies.

SPECULATION

The NMR data presented here indicate that the Arg 42 residue of parkin is located in the Rpn10-binding site. One family of AR-JP patients have a point mutation at position 42 (Terreni *et al*., 2001), in which Arg is substituted with Pro. It is possible that this mutation induces a significant conformational change in the Rpn10-binding site of the parkin Ubl domain, resulting in impaired proteasomal binding by parkin. Indeed, mutant parkin carrying the Arg-42-Pro mutation was extremely difficult to dissolve at a submillimolar concentration for NMR analysis (data not shown); this insolubility might be associated with loss of the correct functional conformation in the mutant form of parkin. We suggest that this defect hampers the formation of an efficient assembly line for protein degradation, and thereby causes the accumulation of parkin substrates in neurons, leading to Parkinson's disease.

METHODS

Protein expression. The DNA fragment encoding the Ubl domain (amino acids 1–76) of human parkin was cloned into the pGEX-6P-3 vector (Amersham Biosciences) with an N-terminal GST moiety. For expression, the plasmid was cotransformed with the pLysS plasmid (Novagen) into the BL21(DE3)CodonPlus *E. coli* strain (Stratagene). For the production of isotopically labelled protein, cells were grown in M9 minimal media containing [^{15}N]NH_4Cl (1 g l^{-1}) and [u-^{13}C]glucose (2 g l^{-1}). The GST-fusion protein was purified from cell lysates using a glutathione-sepharose column. The fusion protein was cleaved by incubation with 3 units of PreScission protease (Amersham Biosciences) for each milligram of GST-fusion protein for 16 h at 4 °C. GST was removed by the application of the digested products onto a second glutathione-sepharose column. Further purification of the protein was carried out using a Superose12 gel-filtration column (Amersham Biosciences). DNA encoding mouse $Rpn10_{196–306}$ was cloned into the pGEX-6P-1 vector. For expression of $Rpn10_{196–306}$, the plasmid was transformed into the *E. coli* BL21(DE3)CodonPlus strain, and cells were grown in Luria–Bertani media. The expression and purification protocols for $Rpn10_{196–306}$ were generally the same as those used for the parkin Ubl domain.

NMR spectroscopy. NMR samples were prepared at a concentration of 0.1 mM in 90% H_2O/10% ^2H_2O (v/v), 50 mM potassium phosphate buffer, 10 mM [$^2H_{10}$]DTT, pH 6.0. All NMR spectra were recorded at 303 K using Bruker DRX800 or DMX500 spectrometers equipped with 5-mm inverse triple-resonance probes with three-axis gradient coils. Backbone and Cβ resonances were assigned sequentially using the following techniques: two-dimensional (2D) ^{1}H-^{15}N HSQC, constant-time-^{1}H-^{13}C HSQC, and 3D HNCA, HN(CO)CA, HNCO, CBCA(CO)NH and CBCANH spectra. Side-chain and Hα assignments were obtained from HBHA(CO)NH, HBHANH, ^{15}N-edited total-correlation spectroscopy (TOCSY), ^{15}N-edited NOESY, ^{13}C-edited NOESY, HCACO, HCCH-COSY and HCCH-TOCSY spectra. Distance restraints for the parkin Ubl domain were obtained by using ^{15}N-edited NOESY and ^{13}C-edited NOESY spectra. For measurements of residual dipolar couplings, the anisotropic medium used was a nematic-phase liquid-crystalline state, induced by bicelle and cetyltriammonium bromide (CTAB)-doped bicelle (Ottiger & Bax, 1998). The final optimized bicelle concentration for both media was 5% (w/w) for 0.1 mM of the parkin Ubl domain. The 2D ^{1}H-coupled ^{1}H-^{15}N HSQC experiments were used to measure the one-bond ^{15}N-^{1}H scalar coupling ($^1J_{NH}$) values in the isotropic state (in the absence of the liquid-crystalline media) and in the anisotropic media (in the presence of the bicelle or CTAB-doped bicelle). Initial estimates for the axial component of the molecular alignment tensor (*Da*) and the rhombicity (*R*) were obtained from the powder-pattern distribution of the overall ^{15}N-^{1}H RDC ($^1D_{NH}$) values (Clore *et al*., 1998). These values were then optimized in a stepwise manner, using the calculated solution-structure of the parkin Ubl domain as described previously (Kikuchi *et al*., 2002). The final values of *Da* and *R* for the parkin Ubl domain were 12 Hz and 0.21, respectively, for the bicelle media, and 18 Hz and 0.42, respectively, for the CTAB-doped bicelle media. Data processing and analysis was carried out using a Silicon Graphics O2 workstation with XWINNMR. The ^{1}H chemical shifts were referenced to external 4,4-dimethyl-4-silapentane-1-sulphonic acid.

Structural determination. Initial calculations were carried out with the NOE-derived inter-proton distance restraints, and with the backbone ϕ and ψ torsion angles restrained by the program TALOS (Cornilescu *et al*., 1999), and hydrogen-bond restraints in the secondary-structure region. The inter-proton restraints were classified into three categories: 1.8–2.7 Å, 1.8–3.5 Å and 1.8–5.0 Å, corresponding to strong, medium and weak NOE intensities, respectively. The hydrogen-bond restraints (two per hydrogen bond) were set to rNH-O = 1.7–2.3 Å and rN-O = 2.7–3.3 Å, according to the ^{1}H-^{2}H exchange rate for the amide protons, TALOS-based secondary structure identification and the backbone NOE. The RDC-derived restraints were used in the SANI modules (Clore *et al*., 1998) for performing a direct refinement against the measured dipolar-couplings with the program CNS version 1.1 (Brünger *et al*., 1998). The calculations were started with extended structures (Nilges *et al*., 1988), and consisted of a torsion angle space dynamics (TAD) measurement, followed by a Cartesian minimization (Stein *et al*., 1997). The TAD consisted of 2,000 molecular dynamics steps of 15 picoseconds, carried out at 50,000 K, and a cooling phase (50,000 steps of 5 femtoseconds each) with annealing temperatures from 50,000 K to 0 K. A second TAD cooling-phase, consisting of 10,000 steps of 2 femtoseconds each, was applied, with annealing temperatures from 500 K to 0 K.

The final energy minimization was performed with the following force constants: 1,000 kcal mol^{-1} $Å^{-2}$ for bond lengths, 500 kcal mol^{-1} rad^{-2} for angles and improper torsions (which served to maintain planarity and chirality), 4 kcal mol^{-1} $Å^{-4}$ for the quartic van der Waals repulsion term, 10 kcal mol^{-1} $Å^{-2}$ for the experimental distance restraints, and 0.2 kcal mol^{-1} Hz^{-2} for the $^1D_{NH}$ RDC restraints. The stereochemical quality of the structures of the parkin Ubl domain were assessed using the program PROCHECK-NMR (Laskowski *et al*., 1996). Graphic figures were generated by the program MOLMOL (Koradi *et al*., 1996).

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

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