Structure of the HECT domain of human WWP2

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WWP2 is a HECT-domain ubiquitin ligase of the Nedd4 family, which is involved in various important biological processes, such as protein degradation, membrane-protein sorting and transportation, the immune response, pluripotency of embryonic stem cells, tumorigenesis and metastasis. The HECT domain provides the intrinsic ubiquitin ligase activity of WWP2. Here, the expression, purification, crystallization and crystallographic analysis of the HECT domain of human WWP2 (HECT^{WWP2}) are reported. HECT^{WWP2} has been crystallized and the crystals diffracted to 2.50 Å resolution. They belonged to space group *P*4₁2₁2 and the structure has been solved *via* molecular replacement. The overall structure of HECT^{WWP2} has an inverted T-shape. This structure displays a high degree of conservation with previously published structures of Nedd4 subfamily members.

1. Introduction

Protein ubiquitination is an important post-translational modification that regulates various cellular processes, such as protein degradation, endocytosis, sorting and transportation of membrane proteins, protein–protein interactions in signal transduction, aspects of the immune response, gene transcription and apoptosis (Chen *et al.*, 2014; Rotin & Kumar, 2009). Ubiquitination occurs through three sequential transfers of ubiquitin (Ub), a 76-residue protein, catalyzed by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugation enzyme (E2) and a ubiquitin ligase (E3) (Hershko & Ciechanover, 1998). In mammals there are approximately 600 genes encoding E3 activity that are responsible for substrate recognition. Based on their domain compositions and substrate-recognition mechanisms, the E3 ligases have been classified into three main families: RING (Really Interesting New Gene), HECT (homologous to the E6AP carboxyl-terminus) and U-box (a type of modified RING motif) (Hutchins *et al.*, 2013). The RING-finger E3s can be further subdivided into (i) the RNF-domain E3s containing a simple RING-finger domain, (ii) the RBR E3s, which contain two RING domains and an IBR domain, and (iii) the CRL ubiquitin ligase complexes, which consist of a cullin protein and a RING protein. The HECT-domain E3 ligases are comprised of those possessing WW domains (the Nedd4/Nedd4-like family) and those lacking double-tryptophan (WW) domains (*e.g.* E6AP). U-box E3 ligases are highly similar to the RING-finger domain proteins in terms of structure. However, U-box E3 ligases themselves bind E2 and ubiquitinate the substrates independently of any other known E3 family members.

Among 28 HECT E3 ligases, the Nedd4/Nedd4-like family, represented by nine members in humans, is composed of an N-terminal C2 domain, two to four WW domains and a

catalytic HECT domain, which has intrinsic ubiquitin ligase activity. Human WWP2 (WW domain-containing E3 Ub-protein ligase 2), also named AIP2 (atrophin-1 interacting protein 2), is a member of the Nedd4/Nedd4-like family. WWP2 contains three recognizable domains: an N-terminal Ca^{2+} /phospholipid-binding C2 domain for membrane/phospholipid binding, four tandem WW domains for substrate recognition and a C-terminal HECT domain for ubiquitin ligation (Bernassola *et al.*, 2008). Three isoforms of WWP2 have been found to date. WWP2-N contains the C2 domain and the first WW domain (WW1), WWP2-C contains the fourth WW domain (WW4) and the HECT domain, and WWP2-FL contains all three domains (Mund *et al.*, 2014).

WWP2 regulates the protein turnover of the epithelial Na^+ channel (ENaC), divalent metal ion transporter 1 (DMT-1), the large subunit of RNA polymerase II (Rpb1) and ADAR2 (adenosine deaminase, RNA-specific, B1) through ubiquitin-dependent degradation (Foot *et al.*, 2008; Li *et al.*, 2007; McDonald *et al.*, 2002). Previous studies have also indicated that WWP2 plays a key role in the regulation of the immune system by ubiquitinating early growth response 2 (EGR2) and TIR-domain-containing adapter-inducing interferon- β (TRIF) (Chen *et al.*, 2009; Yang *et al.*, 2013). In addition, WWP2 enhances octamer-binding transcription factor 4 (OCT4) degradation in differentiated embryonal carcinoma cells (ECCs) rather than undifferentiated ECCs and human embryonic stem cells (ESCs) (Mund *et al.*, 2014; Xu *et al.*, 2004). More recently, WWP2 has been shown to interact with Lys119-methylated SOX2 through its HECT domain to promote SOX2 ubiquitination (Fang *et al.*, 2014). In addition to the crucial roles of WWP2 in the immune and stem-cell systems, it has been demonstrated that WWP2 contributes to various oncogenic signalling pathways through the proteasome-dependent proteolytic degradation of phosphatase and tensin homologue (PTEN), small mothers against decapentaplegic (Smad) and OCT4 (Maddika *et al.*, 2011; Qian *et al.*, 2012; Mund *et al.*, 2014).

Considering its oncogenic functions during tumourigenesis and metastasis, WWP2 stands out as a particularly important ubiquitin ligase. It would be desirable to perform studies on WWP2 in order to develop highly specific WWP2 inhibitors that are able to distinguish WWP2 from other Nedd4 family members. Although a number of HECT-domain structures have been deposited, the WWP2 HECT-domain (HECT^{WWP2}) structure has not yet been reported. We now describe the purification, crystallization and structural analysis of human HECT^{WWP2}.

2. Materials and methods

2.1. Protein expression and purification

The gene encoding full-length human WWP2 was a gift from Jiemin Weng's laboratory at East China Normal University. The corresponding ORF for WWP2 (residues 486–865) was subcloned into a derivative vector of pRSF-Duet, generating a fusion protein with an N-terminal His tag that is

cleavable by PreScission protease. The plasmid, which was verified by DNA sequencing, was transformed into *Escherichia coli* BL21(DE3) competent cells. The transformants were cultivated at 310 K to an OD_{600} of 0.6 in $2\times$ yeast tryptone medium containing 50 mg ml^{-1} kanamycin. Gene expression was induced by adding 0.1 mM isopropyl β -D-1-thiogalactopyranoside. After further incubation at 288 K overnight, the cells were harvested. The cell pellet was resuspended in lysis buffer consisting of 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole supplemented with DNase I. The cells were disrupted at 277 K using a high-pressure homogenizer (Union Biotech). The supernatant was separated from cell debris by centrifugation at 277 K and $31\,000g$ for 35 min (Beckman) and was loaded onto an Ni-NTA affinity column (GE Healthcare). The bound protein was washed with a buffer consisting of 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 25 mM imidazole, followed by on-column digestion with PreScission protease overnight at 277 K. The molecular weight of HECT^{WWP2} is 42.2 kDa , including an additional five-residue peptide (Gly-Pro-Leu-Gly-Ser) derived from the PreScission cleavage site. The eluted protein was loaded onto a Source 15Q anion-exchange column (GE Healthcare) followed by gel-filtration chromatography on Superdex 200 (10/300 GL, GE Healthcare) with a buffer consisting of 50 mM Tris-HCl pH 8.9, 150 mM NaCl, 3 mM TCEP, 5% glycerol. The peak fractions were collected and concentrated to 10 mg ml^{-1} for crystallization.

2.2. Crystallization

Initial crystallization trials were performed with commercial kits, including the Crystal Screen, Index, SaltRx, PEGRx and PEG/Ion kits from Hampton Research and Wizard I–III from Emerald Bio, at 291 and 277 K. The droplets were set up by the hanging-drop vapour-diffusion method, in which $1\text{ }\mu\text{l}$ protein solution was mixed with $1\text{ }\mu\text{l}$ reservoir solution. Crystals suitable for X-ray diffraction were obtained from a condition consisting of 0.1 M HEPES pH 8.4, 0.2 M MgCl_2 , 15% ethanol at 277 K.

2.3. Data collection and processing

All crystals were flash-cooled in liquid nitrogen with cryoprotectant consisting of the crystallization components with an additional 20% glycerol. Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF) using an ADSC Quantum 315 detector. The data were indexed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997).

2.4. Structure solution and refinement

The crystal structure was determined by molecular replacement using *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite (Winn *et al.*, 2011). The structure of the human WWP1 HECT domain (PDB entry 1nd7; Verdecia *et al.*, 2003) was used as the search model. Further modelling was performed using *Coot* (Emsley & Cowtan, 2004). Data anisotropy was detected using *phenix.xtriage* (Adams *et al.*, 2010) and automatically

Table 1

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	ADSC Quantum 315
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	$P4_12_12$
a, b, c (Å)	61.77, 61.77, 242.94
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.56–0.99
Resolution range (Å)	50–2.50 (2.59–2.50)
Total No. of reflections	128397
No. of unique reflections	17210
Completeness (%)	99.9 (100.0)
Multiplicity	7.5 (7.7)
$\langle I/\sigma(I) \rangle$	38.0 (2.9)
Overall B factor from Wilson plot (Å ²)	48.1

corrected using the online server at [*http://services.mbi.ucla.edu/anisotom/*](http://services.mbi.ucla.edu/anisotom/) (Strong *et al.*, 2006). The data after anisotropic correction were used in subsequent refinement with *PHENIX* (Adams *et al.*, 2010), apart from a randomly selected 5% of the reflections which were used for cross-validation. The model was manually rebuilt with *Coot* (Emsley & Cowtan, 2004). All refinements were performed using *phenix.refine* (Afonine *et al.*, 2012). The quality of the final model was validated using *PROCHECK* (Laskowski *et al.*, 1993). The buried surface area was calculated using *PISA* (Krissinel & Henrick, 2007) in the *CCP4* package. All molecular figures were generated using *PyMOL* (DeLano, 2002). Interaction analysis was performed with *LIGPLOT* (Wallace *et al.*, 1995).

3. Results and discussion

3.1. Crystallization, data processing, structure determination and refinement

It has been reported that the C-terminal region of the HECT domain is essential for catalysis, in particular the terminal acidic residue Asp or Glu, which may be in the proximity of the catalytic site (Maspero *et al.*, 2013). In the initial crystal screening the intact HECT domain of WWP2 (residues 486–870) was used, but the protein tended to aggregate at high concentrations. We therefore purified further constructs with various N- and C-terminal truncations for crystallization. The HECT domain of WWP2 (residues 486–865) gave the best crystals for data collection. Crystals with a bipyramidal shape grew in 5 d at 277 K. The dimensions of most of the crystals were about $30 \times 30 \times 100 \mu\text{m}$.

The crystal of HECT^{WWP2} belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 61.77$, $c = 242.94$ Å, $\alpha = \beta = \gamma = 90^\circ$. Data were collected and processed to 2.50 Å resolution. The diffraction pattern of the HECT^{WWP2} crystals suggested the existence of anisotropy, which was confirmed by the observation that the scale factors differed by at least a factor of two in different directions using *phenix.xtriage*

Table 2

Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	50–2.50 (2.59–2.50)
No. of reflections, working set	16298
No. of reflections, test set	857
Final R_{cryst}	0.244 (0.292)
Final R_{free}	0.285 (0.330)
No. of non-H atoms	
Total	2908
Protein	2839
Water	69
R.m.s. deviations	
Bonds (Å)	0.003
Angles (°)	0.648
Average B factors (Å ²)	
Protein	37.1
Water	35.0
Ramachandran plot	
Favoured regions (%)	96.7
Additionally allowed (%)	3.3

(Adams *et al.*, 2010). Therefore, the data were submitted to the online server at [*http://services.mbi.ucla.edu/anisotom/*](http://services.mbi.ucla.edu/anisotom/) (Strong *et al.*, 2006) and this analysis showed strong anisotropy with diffraction along the c^* axis superior to that in the other two directions. After anisotropic correction, the data set was used for molecular replacement and refinement. Data-collection statistics are presented in Table 1 and refinement statistics are presented in Table 2.

Calculation of the Matthews coefficient (Adams *et al.*, 2010; Kantardjieff & Rupp, 2003) suggested that one molecule in an asymmetric unit would give a reasonable V_M ($2.54 \text{ Å}^3 \text{ Da}^{-1}$). The crystal structure of HECT^{WWP2} was determined by molecular replacement using the structure of the human WWP1 HECT domain as a search model (a single molecule derived from PDB entry 1nd7; Verdecia *et al.*, 2003). The sequence identity between the two proteins is 83%. *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite (Winn *et al.*, 2011) was used for structure determination, which generated a distinct peak with a rotational Z -score of 5.4, a translational Z -score of 8.3 and an LLG of 654 in space group $P4_12_12$. The solution was used to position the model, and rigid-body refinement using *REFMAC5* (Murshudov *et al.*, 2011) gave an R factor of 46.4% at 2.50 Å resolution.

Although the anisotropic correction had no obvious effect on the R and R_{free} factors, the quality of the electron-density map was moderately improved. Trials using translation/libration/screw (TLS) restraints failed to improve the electron-density map or to decrease the R and R_{free} factors. Therefore, the final structure was refined to 2.50 Å resolution with an R factor of 24.4% and an R_{free} of 28.5% using the anisotropy-corrected data (Table 2).

3.2. Overall structure of the HECT domain of WWP2

The structure of HECT^{WWP2} shows a domain composed of two lobes (the N-lobe and C-lobe) connected by a flexible hinge (Fig. 1a), which is the typical HECT fold (Huang *et al.*, 1999). The two lobes function cooperatively to bring the E2 enzyme and substrate protein together, mediating the transfer

of ubiquitin from E2 to the HECT domain and then to the target protein (Verdecia *et al.*, 2003). The N-lobe consists of large and small subdomains. The large subdomain of the N-lobe (coloured dark green) is composed of nine α -helices ($\alpha 1$ – $\alpha 6$ and $\alpha 10$ – $\alpha 12$) and four β -strands ($\beta 1$ – $\beta 4$). The small subdomain (coloured light green) is composed of one helix ($\alpha 7$) and two antiparallel helices ($\alpha 8$ and $\alpha 9$). Notably, residues 661–702 of WWP2 were not built in the model owing to a lack of electron density, which might be the result of intrinsic flexibility. This region is believed to be the E2 binding site of the HECT-domain E3 ligases. The C-lobe is an α/β -sandwich domain which is comprised of helices $\alpha 13$ – $\alpha 16$ and strands $\beta 5$ – $\beta 8$, containing a highly conserved catalytic cysteine (Cys838) (Fig. 1*b*).

3.3. Structural comparison of the HECT domains with other Nedd4 E3 ligases

We performed structural comparison between HECT^{WWP2} (residues 486–865) and HECT^{WWP1} (residues 544–917). The overall structure of HECT^{WWP2} presents a similar conformation, as shown in Supplementary Fig. S1(*a*). A structure-based sequence alignment of Nedd4 family members is shown in Supplementary Fig. S1(*b*); HECT^{WWP2} shows 83% sequence similarity to HECT^{WWP1}.

Structures of the HECT domain present in other proteins have been deposited in the PDB. Structural comparison shows

that two different groups of overall fold, an inverted T-shape and an L-shape, could be assigned according to the relative positions of the C-lobe and the N-lobe (Supplementary Fig. S2). This conformational difference is reported to be owing to rotation around the hinge loop (residues 751–754 in WWP2; Verdecia *et al.*, 2003). The HECT domains of Nedd4/Nedd4-like family members, such as WWP1 (Verdecia *et al.*, 2003), Nedd4L, HUWE1 (Pandya *et al.*, 2010) and Itch, as well as WWP2, adopt an inverted T-shape in which the C-lobe binds to the middle of the N-lobe (Supplementary Fig. S2*a*). Among the inverted T-shape structures, the overall C α maximum r.m.s.d. of HECT^{WWP2} (321 residues) is 7.96 Å from HECT^{Nedd4L} (321 residues), as calculated using the MUSTANG server (Konagurthu & Lesk, 2010). The other r.m.s.d. values are 1.14, 1.74 and 1.65 Å when compared with WWP1, HUWE1 and Itch, respectively. In contrast, the HECT domains of Smurf2 (Ogunjimi *et al.*, 2005) and Nedd4 (Maspero *et al.*, 2011) adopt the L-shape, in which the C-lobe binds close to the end of the N-lobe (Supplementary Fig. S2*b*). The buried surface area (1827 Å²) between the C-lobe and the N-lobe in WWP2 (inverted T-shape) is much larger than that (1048 Å²) in Nedd4 (L-shape), suggesting that the HECT domain is more stable in the inverted T-shape than in the L-shape (Verdecia *et al.*, 2003). Intriguingly, previous studies suggest that association of ubiquitin leads to C-lobe rotation around the hinge and conformational change of Nedd4 from the L-shape to the inverted T-shape (Maspero *et al.*, 2013).

Figure 1

Overall structure of HECT^{WWP2}. (*a*) Colour-coded domain architecture of HECT^{WWP2}. The large subdomain of the N-lobe is dark green, the small subdomain is light green, the hinge region is orange and the C-lobe is purple. (*b*) Ribbon representations of the HECT^{WWP2} structure are shown in two different views, and the secondary-structure composition is listed below.

However, the conformation of the ternary complex $\text{Rsp5}^{\text{WW3-HECT}}\text{-Ub-Sna3}^{\text{C}}$ still retains the L-shape as in $\text{HECT}^{\text{Rsp5}}$ (Kim *et al.*, 2011; Kamadurai *et al.*, 2013).

3.4. Analysis of N- and C-lobe interaction in HECT domains

The relative positions of the N- and C-lobes in $\text{HECT}^{\text{WWP2}}$ resemble those of WWP1 , Nedd4L , HUWE1 and Itch , and obviously differ from those of Smurf2 and Nedd4 . The two conformational states utilize different sets of conserved residues to mediate the bilobal interaction. Compared with $\text{HECT}^{\text{Nedd4}}$, the overall structure of $\text{HECT}^{\text{WWP2}}$ is more compact owing to the extensive interaction established between the N- and C-lobes of $\text{HECT}^{\text{WWP2}}$ (Fig. 2*a*). In the L-shaped Nedd4 , Tyr616 in the N-lobe forms a weak hydrogen

bond to Ser817 in the C-lobe with a distance of 3.4 Å. The two lobes of $\text{HECT}^{\text{Nedd4}}$ have no stronger interaction at the interface (Fig. 2*b*). However, Lys642 and Glu645 from helix $\alpha 8$ of $\text{HECT}^{\text{WWP2}}$ form two hydrogen bonds to Ser835 and His836 of strand $\beta 7$, respectively. Asp741, Lys743 and Glu746 residing at the terminus of the N-lobe form extra three hydrogen bonds to Arg793, Gln796 from helix 15 α and Thr801. Additionally, the acylamino group of Gln796 forms a hydrogen bond to the carbonyl O atom of Lys743. Moreover, the conserved residue Tyr587 (equivalent to Tyr616 in Nedd4) forms another hydrogen bond to Thr837 with a distance of 3.24 Å (Fig. 2*c*).

$\text{HECT}^{\text{WWP2}}$ and $\text{HECT}^{\text{WWP1}}$ both adopt an inverted T-shape, and there are some similarities and differences between the N-lobe and C-lobe interactions. In $\text{HECT}^{\text{WWP1}}$ Glu798 forms hydrogen bonds to Gln848 and Thr853, and

Figure 2

Structural superposition of $\text{HECT}^{\text{WWP2}}$ with the three-dimensional structure of $\text{HECT}^{\text{Nedd4}}$. (a) Cartoon representation of superposition of $\text{HECT}^{\text{WWP2}}$ (N-lobe of $\text{HECT}^{\text{WWP2}}$, green; C-lobe of $\text{HECT}^{\text{WWP2}}$, blue) and $\text{HECT}^{\text{Nedd4}}$ (grey). (b) Enlargement of the N- and C-lobe interaction interface of $\text{HECT}^{\text{Nedd4}}$. The residue of the N-lobe involved in interaction is coloured magenta and that of the C-lobe is coloured yellow. All of the residues are highlighted in stick representation. The hydrogen bond is shown as a dashed line. (c) Enlargement of the N- and C-lobe binding interface of $\text{HECT}^{\text{WWP2}}$. The N- and C-lobes of $\text{HECT}^{\text{WWP2}}$ are represented as cartoons. The N-lobe is coloured green and the C-lobe is coloured blue. Residues involved in the interaction of the N- and C-lobes are highlighted in stick representation. Hydrogen bonds are shown as dashed lines.

Figure 3

Comparison of the N- and C-lobe interaction interface between HECT^{WWP1} and HECT^{WWP2}. (a) Cartoon representation of HECT^{WWP1} (N-lobe of HECT^{WWP1}, yellow; C-lobe of HECT^{WWP1}, magenta). Residues involved in N- and C-lobe interaction are highlighted in stick representation. Hydrogen bonds are shown as dark dashed lines and the disulfide bond is shown in yellow. (b) Enlarged superposition cartoon of the N- and C-lobe interface of HECT^{WWP1} and HECT^{WWP2} (N-lobe of HECT^{WWP2}, green; C-lobe of HECT^{WWP2}, blue). Residues involved in the interaction of the N- and C-lobes of HECT^{WWP2} are labelled in green. All residues are highlighted in stick representation.

Asp793 forms hydrogen bonds to Arg845 (Fig. 3a), which are spatially equivalent to those from Glu746 to Gln796 and Thr801 and from Asp741 to Arg793 in HECT^{WWP2} (Fig. 3b). Previous studies demonstrated that mutation of Glu798 and Gln848 in WWP1 completely abolished the ubiquitin-transfer activity (Verdecia *et al.*, 2003). We speculate that residues Glu746 and Gln796 in WWP2 are also closely related to its ubiquitination activity. In HECT^{WWP1}, in addition to these hydrogen-bond interactions, Cys802 bridges to Cys854 through a disulfide bond, and the carbonyl O atom of Thr676 forms a hydrogen bond to the side chain of Arg855 (Fig. 3a). Although these residues are highly conserved in WWP2, these interactions are not found in the HECT^{WWP2} structure. Conversely, the interactions between helix $\alpha 8$ and strand $\beta 7$ of HECT^{WWP2} do not exist in HECT^{WWP1} (Fig. 3b).

Viewed as a whole, we infer that although the HECT domains of the Nedd4 family share highly conserved residues, the members present different conformational states. Furthermore, the relative position of the two lobes is determined by different molecular interactions in the inverted T-shape.

4. Conclusions

We determined the crystal structure of the HECT domain of WWP2 at 2.50 Å resolution. The HECT domain adopts an inverted T-shape conformation, which is the representative architecture of Nedd4 subfamily members. The N- and C-lobes of HECT^{WWP2} interact with each other through several hydrogen-bonding interactions to maintain a more compact conformation. Nedd4 family members employ distinctive interactions to maintain their conformational state. Since WWP2 has emerged as multifunctional protein that is able to

target a series of proteins involved in oncogenic signalling pathways, this crystal structure may provide a foundation for structure-based drug design.

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