

Recognition of the *iso*-ADP-ribose moiety in poly(ADP-ribose) by WWE domains suggests a general mechanism for poly(ADP-ribosylation)-dependent ubiquitination

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Protein poly(ADP-ribosylation) and ubiquitination are two key post-translational modifications regulating many biological processes. Through crystallographic and biochemical analysis, we show that the RNF146 WWE domain recognizes poly(ADP-ribose) (PAR) by interacting with *iso*-ADP-ribose (*iso*-ADPR), the smallest internal PAR structural unit containing the characteristic ribose-ribose glycosidic bond formed during poly(ADP-ribosylation). The key *iso*-ADPR-binding residues we identified are highly conserved among WWE domains. Binding assays further demonstrate that PAR binding is a common function for the WWE domain family. Since many WWE domain-containing proteins are known E3 ubiquitin ligases, our results suggest that protein poly(ADP-ribosylation) may be a general mechanism to target proteins for ubiquitination.

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Protein ubiquitination regulates diverse biological processes; however, the mechanism by which proteins are earmarked for ubiquitination remains incompletely understood. Other than phosphorylation, which is a general mechanism for many cases, hydroxylation of a substrate (i.e., HIF1- α) and the binding of small molecules (e.g., the plant hormone auxin) to E3 ligases have been shown to control protein ubiquitination in sporadic cases (Willems et al. 1999; Min et al. 2002; Bergink and Jentsch 2009; Tan and

Zheng 2009). Protein poly(ADP-ribosylation) (PARylation), catalyzed by PAR polymerases (PARPs), also regulates a myriad of biological processes, including DNA damage responses, transcriptional regulation, intracellular trafficking, energy metabolism, circadian rhythm, and cell survival and cell death programs, among others (Curtin 2005; Jagtap and Szabo 2005; Kim et al. 2005; Schreiber et al. 2006; Krishnakumar and Kraus 2010). How PARylation affects so many biological functions remains largely mysterious. In many cases, such as PARylation of histones in transcriptional regulation, PARylation is considered to control activities of the substrate proteins via the negative charges in the PAR polymer (Schreiber et al. 2006; Krishnakumar and Kraus 2010). In other cases, PAR polymers have been implicated as signaling molecules that can induce cell death, especially in the brain (Andrabi et al. 2006, 2011).

Most recently, PARylation has been shown to control the polyubiquitination and degradation of axin, a key regulator of the Wnt signaling pathway (Huang et al. 2009; Callow et al. 2011; Kang et al. 2011; Zhang et al. 2011). In all of these reports, RNF146 (aka Iduna), which contains a WWE domain and a RING domain (Supplemental Fig. 1), is the only known E3 ubiquitin ligase to date that requires PARylation of the substrate for subsequent polyubiquitination (Callow et al. 2011; Kang et al. 2011; Zhang et al. 2011). The RNF146 WWE domain has been shown to bind PAR (Callow et al. 2011; Zhang et al. 2011), and it was reported that a short PAR-binding motif (PBM) within the domain retains this binding activity (Andrabi et al. 2011). The PBM was originally found in histones and several other proteins (Gagne et al. 2008). However, the PBM identified in RNF146 is not conserved in other WWE domains, so it remains unclear whether the WWE domain represents a novel PAR-binding domain. Here we reveal the structural basis of the RNF146 WWE domain/*iso*-ADPR interaction and, for the first time, define the PAR/*iso*-ADPR binding as a bona fide function of the WWE domain family. Importantly, the structural coupling of WWE domains and E3 ligase domains in many WWE domain-containing proteins suggests a functional coupling of protein PARylation and ubiquitination.

Results and Discussion

The RNF146 WWE domain recognizes iso-ADPR, but not ADPR

We sought to clarify the requirement of the entire RNF146 WWE domain structure for PAR binding through structural analysis. PAR polymers display high chemical heterogeneity (in both lengths and branching patterns) and are not suitable for quantitative and structural analysis. Thus, we first examined its interaction with ADP-ribose (ADPR), the building unit added to PAR during PAR synthesis (Fig. 1A). Isothermal titration calorimetry (ITC) analysis demonstrated that the RNF146 WWE domain does not interact with ADPR, even at high concentrations (>0.1 mM) (Fig. 1C). We then turned to *iso*-ADPR, which is the smallest PAR structural unit containing the ribose-ribose glycosidic bond unique to PAR, formed during PAR synthesis by PARPs (Fig. 1A). It remains a major challenge to obtain PAR of a specific length in sufficient quantities

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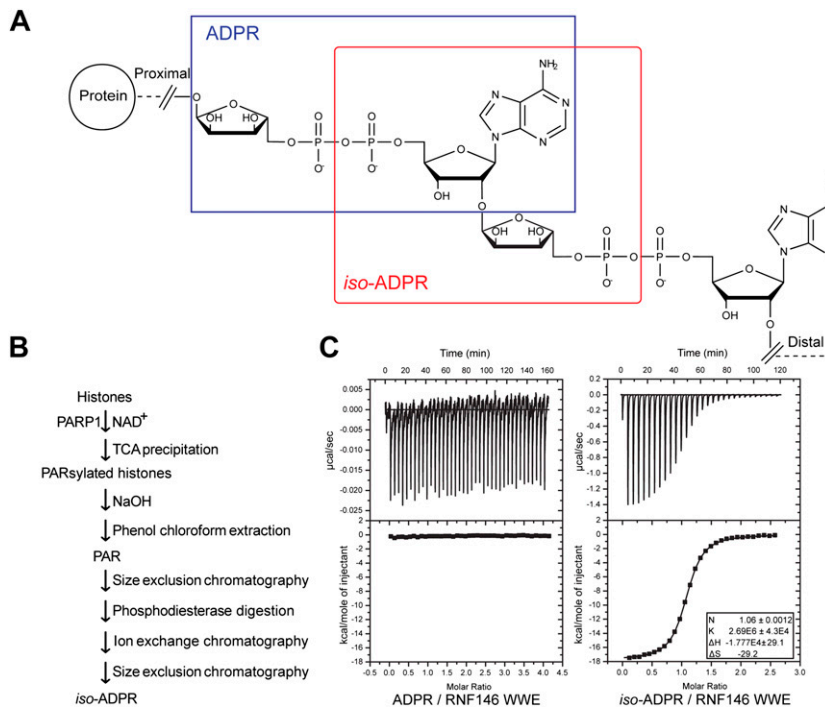


Figure 1. The interaction between the RNF146 WWE domain and PAR, through the recognition of *iso*-ADPR. (A) Structures of PAR and its structural units. ADPR is the unit within the blue frame, and *iso*-ADPR is within the red frame. (B) Procedural outline of *iso*-ADPR in vitro biosynthesis and purification. (C) ITC analysis showed the binding of the RNF146 WWE domain with *iso*-ADPR ($K_d \sim 370$ nM), but not with ADPR.

for biochemical analysis, and *iso*-ADPR is not commercially available, nor has it been used previously in structural and biochemical studies. We therefore developed a protocol to biosynthetically generate *iso*-ADPR (Fig. 1B). We synthesized PAR using histone PARylation by PARP1, following previously published protocols (Kiehlbauch et al. 1993; Fahrner et al. 2007). After removing small molecules by size exclusion chromatography (SEC), we digested PAR polymers with a phosphodiesterase to generate *iso*-ADPR. Finally, we purified *iso*-ADPR by ion exchange and a second SEC step to remove all remaining large molecules. The purity and identity of purified *iso*-ADPR were confirmed by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. ITC analysis demonstrated that, in contrast to the poor interaction with ADPR, the RNF146 WWE domain interacted with *iso*-ADPR avidly, with a dissociation constant of $0.37 \mu\text{M}$ (Fig. 1C). Selectivity of the RNF146 WWE domain for *iso*-ADPR was also demonstrated by its comigration with *iso*-ADPR, but not ADPR, in SEC (Supplemental Fig. 2). Thus, the RNF146 WWE domain interacts with the PAR polymer, but not mono-ADPR. This is likely to be important for RNF146 function, since ADP-ribosylation and PARylation are performed by different enzymes and have distinct biological functions (Okazaki and Moss 1996; Corda and Di Girolamo 2002; Curtin 2005; Jagtap and Szabo 2005; Schreiber et al. 2006; Krishnakumar and Kraus 2010). We note that the ability to generate purified *iso*-ADPR provides a unique reagent for biochemical and structural analysis of PAR-binding proteins and mechanistic analysis of PAR-metabolizing enzymes, such as PARG that cleaves the glycosidic bond.

Crystal structure of the RNF146 WWE domain in complex with *iso*-ADPR

To understand how the RNF146 WWE domain interacts with *iso*-ADPR, we determined the crystal structure of the RNF146 WWE domain in complex with *iso*-ADPR at 1.63 \AA resolution (Fig. 2A,B; Supplemental Table 1). The WWE domain contains six β strands, forming half of a β barrel, with the other side of this half β barrel covered by an α helix. The high resolution of our structure allowed us to define unambiguously that the ribose-ribose linkage in *iso*-ADPR is an $\alpha(1 \rightarrow 2)$ glycosidic bond (Fig. 2B). This confirmed that PAR synthesis catalyzed by PARP1 is $\alpha(1 \rightarrow 2)$ -specific. The adenine ring of *iso*-ADPR inserts into the pocket formed by the half β barrel and the α helix (Fig. 2A). The two ribose-phosphate moieties on both sides of *iso*-ADPR sit on the edge of the half β barrel, which is highly positively charged (Fig. 2C). Both sides of *iso*-ADPR, especially the two separated phosphate groups, are involved in extensive interactions with WWE domain residues (Fig. 2D), providing an explanation for why the RNF146 WWE domain specifically binds to *iso*-ADPR and thus to PAR, but not ADPR, which has the two phosphate groups on the same side (Fig. 1A). Compared with a previously determined nuclear magnetic resonance (NMR) structure of an unliganded RNF146 WWE domain (Protein Data Bank [PDB] code 1UJR), it appears that *iso*-ADPR binding induces significant conformational changes in the WWE domain, particularly in the C-terminal tail region (residues 169–183), which folds back to support the distal ribose-phosphate groups of *iso*-ADPR (Supplemental Fig. 3). The mode of binding displayed in our crystal structure, in which the two phosphate groups lie on an open surface of the RNF146 WWE domain, should allow internal *iso*-ADPR units in PAR polymers to bind the WWE domain in the same manner.

Mutagenesis analysis of the RNF146 WWE domain

Seven RNF146 WWE domain surface residues are involved in *iso*-ADPR binding (Fig. 2D). Among them, the Tyr 107 phenol group stacks on the side of the *iso*-ADPR adenine ring within the pocket, and Gln 153 near the bottom of the adenine-binding pocket forms two hydrogen bonds with the adenine ring and appears to confer binding specificity as well as binding affinity. The Arg 163 and Tyr 144 side chain groups interact with the proximal phosphate group, and Trp 114, Arg 110, and Lys 175 interact with the ribose-phosphate groups in the distal side of *iso*-ADPR (Fig. 2D). To validate our structure and define key interactions between the RNF146 WWE domain and *iso*-ADPR, we analyzed the *iso*-ADPR binding of seven RNF146 WWE domain mutants by ITC analysis. Mutants Y107A, Y144A, and R163A lack a detectable interaction, and mutants W114A and Q153A have much lower affinity than the

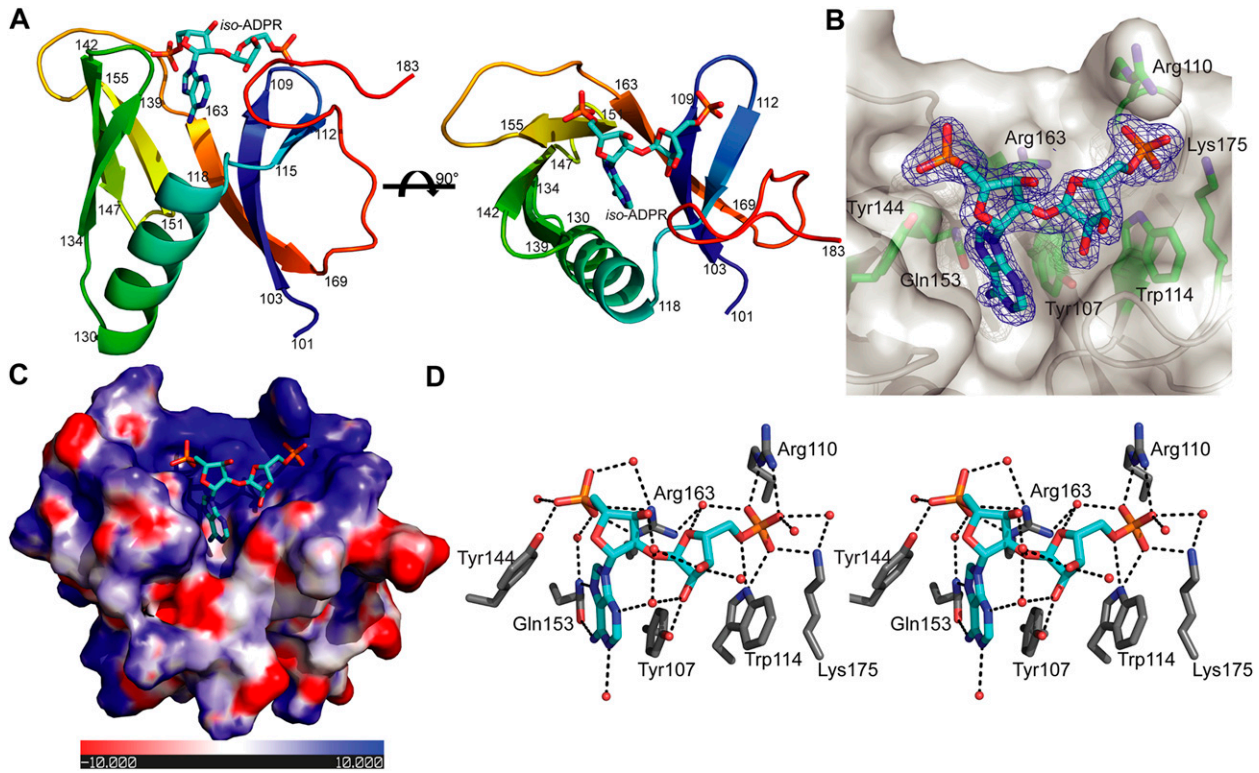


Figure 2. Crystal structure of the RNF146 WWE domain in complex with *iso*-ADPR. (A) Overall structure of the complex. The protein is shown in rainbow, and *iso*-ADPR is shown in cyan. (B) $F_o - F_c$ difference density (blue mesh) calculated when *iso*-ADPR is omitted (contoured at 2.5σ). Protein is shown in gray, and key binding residues are highlighted in sticks. (C) The electrostatic surface potentials of the *iso*-ADPR-binding region, calculated using Pymol. Negative potential patches are shown in red, and positive potential patches are shown in blue. (D) Stereoview of key interactions (in black dash lines) involved in *iso*-ADPR binding with the RNF146 WWE domain. Key binding residues are highlighted in sticks, and water molecules are shown as pink spheres.

wild-type RNF146 WWE domain. The mutations R110A and K175A had only a minor effect on the interaction (summarized in Fig. 3A, Supplemental Fig. 4). Then we tested the binding of RNF146 mutants to PAR in the context of a full-length protein. Consistent with the ITC analysis using the WWE domain, the RNF146 mutants Y107A, Y144A, R163A, and Q153A all lost the ability to interact with PAR in a coimmunoprecipitation assay (Fig. 3B).

To demonstrate the importance of the RNF146 WWE domain residues involved in PAR binding, we examined the effect of their mutation in full-length RNF146 protein on axin turnover in a cellular context. Expression of siRNA-

resistant RNF146 completely rescued the effect of RNF146 siRNA on Axin1, whereas expression of RNF146 mutants failed to do so (Fig. 3C). These experiments confirm that the specific interactions observed in the crystal structure are important for the *in vivo* functions of RNF146.

The previously proposed PBM in RNF146 (residues 144–167) (Andrabi et al. 2011) only accounts for three β strands in the WWE domain structure (Fig. 2A). Residues outside the PBM region interact extensively with *iso*-ADPR (Fig. 2C,D), and missense mutations of the RNF146 WWE domain outside the PBM, such as Y107A, abolish the PAR-binding activity (Fig. 3). Therefore, we conclude that

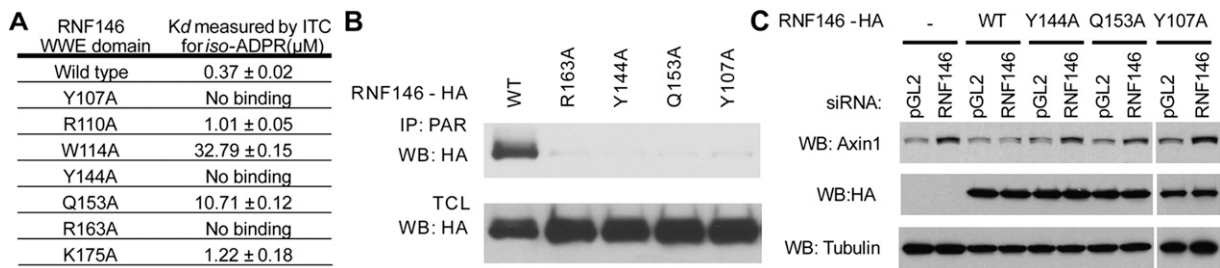


Figure 3. Mutagenic analysis of crucial WWE domain residues involved in *iso*-ADPR binding. (A) Dissociation constants of RNF146 WWE domain mutants with *iso*-ADPR as measured by ITC analysis. (B) Coimmunoprecipitation of wild-type RNF146—but not RNF146 mutants Y107A, Y144A, Q153A, and R163A—by anti-PAR antibody in HEK293 cells. (C) Expression of siRNA-resistant wild-type RNF146—but not RNF146 mutants Y107A, Y144A, and Q153A—rescues RNF146 siRNA-induced stabilization of Axin1 in HEK293 cells.

the RNF146 WWE domain is a bona fide PAR-binding domain, which specifically recognizes the *iso*-ADPR moiety of PAR.

PAR/*iso*-ADPR binding is a common function of WWE domains

The WWE domain family exhibits a low degree of sequence homology (Fig. 4A). The most conserved residues include the two Trp and one Glu residues that give rise to the name “WWE” domain. These residues are involved in stabilizing the WWE domain fold (Supplemental Fig. 5). Based on the crystal structure of the RNF146 WWE domain/*iso*-ADPR complex and our mutagenesis analysis, we note that critical residues for *iso*-ADPR and PAR binding are conserved in most WWE domains, suggesting that PAR binding may be a common function of the WWE domain family (Fig. 4A). Surface plasma resonance (SPR) analysis demonstrated that GST-tagged WWE domains from HUWE1, ULF, Deltex1, and PARP11 bind to the PAR polymers, whereas the DDHD2 WWE domain did not interact with PAR (Fig. 4B). This is in perfect concordance with our structure and sequence alignment. While the four crucial residues involved in *iso*-ADPR binding in the RNF146 WWE domain are conserved among most WWE domains in the human genome, including these from RNF146, HUWE1, ULF, Deltex1, Deltex2, Deltex4, and PARP11, residues corresponding to RNF146 Q153 and R163 are not conserved in DDHD2 and PARP14 (Fig. 4A).

Given the sequence conservation, we predict that other PAR-binding WWE domains also recognize *iso*-ADPR in the same way as the RNF146 WWE domain. While many of the WWE domains we tested were not suitable for ITC analysis due to aggregation, our ITC analysis with the HUWE1 WWE domain showed that this domain interacts with *iso*-ADPR with a K_d of 13 μ M (Fig. 4C). The interactions between WWE domains and PAR are further enhanced by the high local concentrations of *iso*-ADPR around PAR, as there can be as many as \sim 200 units in each PAR chain. Deltex1/2/4 proteins contain two tandem WWE domains. Structural modeling based on the unliganded *Drosophila* Deltex WWE domain structure (Zweifel et al. 2005) also suggests that these two WWE domains may recognize two neighboring *iso*-ADPR units, with each interacting in a manner similar to the RNF146 WWE domain (Supplemental Fig. 6). Therefore, with the exception of DDHD2 and PARP14, PAR binding through the recognition of *iso*-ADPR is a common function for most WWE domains.

Structural and functional coupling of protein PARylation and ubiquitination

It has been previously noticed that the majority of WWE domains are structurally coupled with E3 ubiquitin ligases (Supplemental Fig. 1; Aravind 2001). Here we show

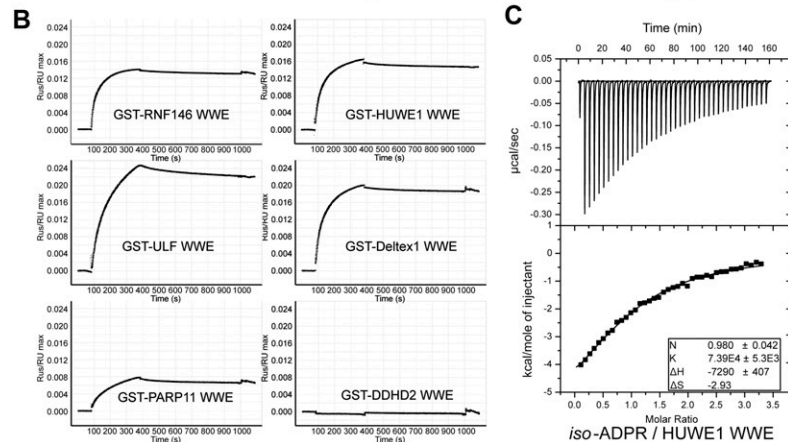
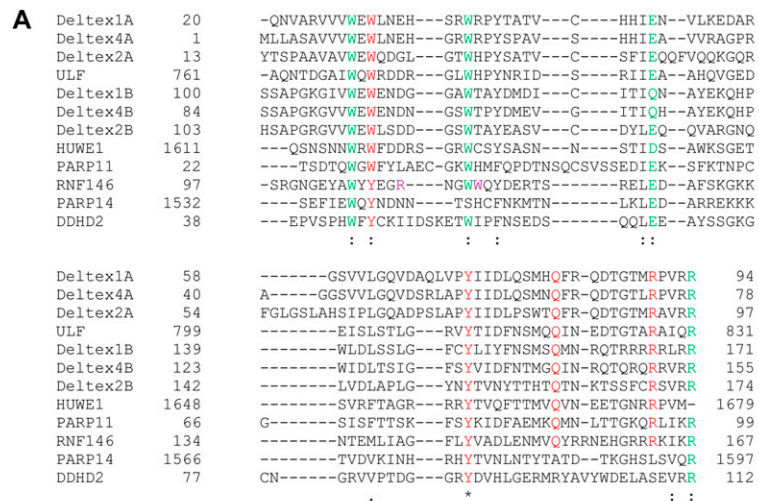


Figure 4. PAR-binding properties of the WWE domain family. (A) Multiple sequence alignment by ClustalW of known human WWE domain sequences. Most conserved residues important for WWE domain folding (see Supplemental Fig. 5) are shown in green. Highly conserved residues potentially involved in *iso*-ADPR binding (corresponding to Tyr 107, Tyr 144, Gln 153, and Arg 163 in RNF146) are highlighted in red, and two other nonconserved *iso*-ADPR-binding residues in RNF146 (Arg 110 and Trp 114) are in purple. The sequence surrounding RNF146 Lys 175 in the C-terminal tail is not conserved and is not shown here. In accordance with the mutagenesis results shown in Figure 3, conserved residues are important for *iso*-ADPR binding, whereas nonconserved residues are not. Deltex proteins contain tandem WWE domains (A and B represent the N-terminal and C-terminal WWE domains, respectively). (B) SPR analysis of the interactions between various GST-tagged WWE domains and PAR. Except for DDHD2, all other WWE domains tested interact with PAR. (C) ITC analysis of the interaction between the HUWE1 WWE domain and *iso*-ADPR.

that the WWE domains from all four E3 domain-containing proteins tested (RNF146, HUWE1, ULF, and Deltex1) interact specifically with PAR. HUWE1 [aka Mule, ARF-BP1, LASU1, and HectH9] is a HECT-type E3 ligase critical for cell regulation; it ubiquitinates the tumor suppressor p53, core histones, and the Bcl-2 family member Mcl-1 (Chen et al. 2006; Bernassola et al. 2008). ULF/TRIP12 is an E3 ubiquitin ligase of ARF, a key activator of p53 (Chen et al. 2010). The Deltex family, which plays an important role in Notch signaling, contains a RING domain and two tandem WWE domains (Zweifel et al. 2005; Katoh 2007). The definition of WWE domains as a novel PAR-binding domain and the structural coupling of the WWE domain with E3 domains suggest that PAR may be a signal for protein ubiquitination—either poly-

ubiquitination that may lead to protein degradation by proteasome, or divergent monoubiquitination that controls protein activities in the cell. The WWE domains may therefore be a key link between protein PARylation and ubiquitination. Furthermore, since the small WWE domains recognize only one (or two) internal *iso*-ADPR unit, numerous WWE domain-containing proteins may potentially cluster around a PAR polymer, a property that may have functional importance. The role of PARPs and PARylation is well established in many biological processes, including DNA repair. The insights provided by the studies reported here will facilitate design of specific mutations in WWE domain-containing E3s that can be used to unravel the role and the molecular mechanisms of PARylation in biological systems. In summary, protein PARylation may be another general mechanism to label proteins for ubiquitination other than protein phosphorylation, and many of the protein PARylation events may function through ubiquitination. Our studies have not only identified the *iso*-ADPR as the critical signaling unit in PAR, but also suggested a role for the WWE domains in a superfamily of ubiquitin ligases in decoding the protein PARylation signal.

Materials and methods

Generation and purification of *iso*-ADPR

Based on the methods reported previously (Kiehlbauch et al. 1993; Fahrner et al. 2007), PAR polymer was synthesized *in vitro* with some modifications. The histone PARylation reaction was set up in a 15-mL incubation mixture comprising 50 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 50 mM NaCl, 10 mM DTT, 1.5 mM β-NAD⁺, 0.1 mg/mL histone H1 (Millipore), 0.1 mg/mL histone type IIA (Sigma-Aldrich), 67 mg/mL activator oligonucleotide GGAATTC, and 5000 U of human PARP-1 (Trevigen). The reaction was stopped after 1.5 h at room temperature by ice-cold trichloroacetic acid. The white pellet was washed with ice-cold ethanol and was further dissolved in buffer containing 0.2 mg/mL Protease K (Invitrogen) and 0.1% SDS. PAR polymer was detached using NaOH and was purified by phenol/chloroform extraction and isopropanol precipitation. After removing small molecules by Superdex 75 on fast protein liquid chromatography (FPLC) (GE healthcare), we digested purified PAR polymer by 50 U of snake venom phosphodiesterase (Worthington) with 15 mM MgCl₂ overnight at room temperature. The product of the phosphodiesterase digestion, *iso*-ADPR, was further purified by anion exchange chromatography and Superdex 75 on FPLC (GE Healthcare). Purified *iso*-ADPR was air-dried and dissolved by ddH₂O to 40 mM final concentration and stored at -20°C. LC-MS detected *m/e* 558.1 [M-H]⁻ with high purity (the calculated mass of *iso*-ADPR in acidic form is 559.1).

Protein purification, crystallization, and structure determination

WWE domains were purified as GST fusion proteins, and the GST tag was removed for crystallographic analysis. Purified Se-Met RNF146 WWE domain (residues 99–183) at 10 mg/mL was mixed at 1:1.5 molar ratio with purified *iso*-ADPR and incubated for 30 min on ice prior to cocrystallization. The hanging-drop method was used to prepare crystals of the Se-Met RNF146 WWE domain in complex with *iso*-ADPR. One microliter of protein-ligand mixture solution was mixed with 1 μL of well solution containing 45% PEG 400, 100 mM Tris-HCl (pH 8.0), and 10 mM DTT. Ship-shaped crystals usually appeared in 1 d at 22°C and grew to their full sizes in 3 d. The crystals were directly flash-frozen in liquid nitrogen. Screening and data collection were performed at the Advanced Light Source (ALS), beamline 8.2.1. All diffraction data were processed by HKL2000 (Otwinowski and Minor 1997). The structure was determined by single-wavelength anomalous dispersion (SAD) using one data set collected at wavelength 0.9793 Å, which was also used for refinement (Supplemental Table 1). The selenium sites and the initial phases were determined by PHENIX (Adams et al. 2010). Four selenium sites were found in

one asymmetric unit, and the experimental electron density map clearly showed the presence of two WWE molecules with two ligands in one asymmetric unit. The complex model was improved using iterative cycles of manual rebuilding with the program COOT (Emsley et al. 2010) and refinement with Refmac5 of the CCP4 6.1.2 program suite (Collaborative Computational Project, Number 4 1994). There is no Ramachandran outlier (98.1% most favored, 1.9% allowed). The electrostatic potential surfaces shown were generated by the APBS tool in Pymol (DeLano and Brunger 1994).

ITC

ITC analyses were carried out using a VP-ITC Microcal calorimeter (MicroCal) at 30°C for the RNF146 WWE domain and its mutants and at 16°C for the HUWE1 WWE domain. All proteins underwent buffer exchange to 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM DTT by a Superdex 75 column, with a final concentration of ~20 μM. Ligands (ADPR and *iso*-ADPR) were also diluted by the same buffer to ~500 μM. A typical titration consisted of injecting 30–40 5-μL aliquots of the ligand into the protein sample (1.4218-mL chamber) at time intervals of 4 min, to ensure that the titration peak reached the baseline. The ITC data were analyzed using the software Origin 7.0 provided by the manufacturer. Data were fit by a one-site model.

SPR

GST-tagged proteins were coupled to Biacore CM5 sensor chip coated with anti-GST antibody. PAR (625 nM; Trevigen) was then profiled at a flow rate of 30 mL/min for 300 sec, followed by a 600-sec flow of wash buffer. After analysis in BiaEvaluation (Biacore), the normalized resonance units were plotted over time with the assumption of 1:1 binding.

Immunoblotting and immunoprecipitation

The RNF146 cDNA rescue experiment and the coimmunoprecipitation experiment were performed as described previously (Zhang et al. 2011). Details of experiments can be found in the Supplemental Material.

Accession number

Coordinates and structure factors have been deposited in the PDB (<http://www.rcsb.org/pdb>) under ID code 3V3L.

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