Structural and Biochemical Basis for Ubiquitin Ligase Recruitment by Arrestin-related Domain-containing Protein-3 (ARRDC3)*

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Background: ARRDC proteins bind ubiquitin ligases and are involved in receptor down-regulation.

Results: ARRDC3 recruitment of Nedd4 is explained.

Conclusion: The first PPXY motif of ARRDC3 binds to the third WW domain of Nedd4 with high affinity, whereas other domains also contribute.

Significance: The structure explains aspects of high affinity recognition.

After protracted stimulation, the β2-adrenergic receptor and many other G-protein-coupled receptors are ubiquitinated and down-regulated. Arrestin-related domain-containing protein-3 (ARRDC3) has been proposed to recruit the ubiquitin ligase Nedd4 to the β2-adrenergic receptor. ARRDC3 contains two PPXY motifs that could potentially interact with any of the four WW domains of Nedd4. Here we dissect the interaction determinants. ARRDC3 PPXY-Nedd4 WW dissociation constants vary from unmeasurable to $K_d = 3 \mu M$ for the third WW domain of Nedd4 binding to the first PPXY motif of ARRDC3. Structures of the uncomplexed and PPXY$_1$-bound WW3 domain were determined at 1.1 and 1.7 Å resolution. The structures revealed conformational changes upon binding and the hydrogen bonding network in exquisite detail. Tight packing of ARRDC3 Val-352’, part of a 3$_{10}$ helix at the C terminus of PPXY$_1$, is important for high affinity binding to WW3. Although no single WW domain is strictly essential for the binding of Nedd4 and ARRDC3 expressed in HEK293 cells, high affinity binding of full-length ARRDC3 and Nedd4 is driven by the avid interaction of both PPXY motifs with either the WW2-WW3 or WW3-WW4 combinations, with $K_d$ values as low as 300 nM.

In normal physiology no cell surface receptor remains indefinitely in an active signaling state. Various mechanisms turn receptor signals off, operating with differing kinetics (1). On short time scales, phosphorylation desensitizes active receptors (2). As long as the receptor remains at the cell surface and is not subjected to further post-translational modification, it can be quickly re-activated by dephosphorylation. In many cases, receptor activation and/or phosphorylation is followed by endocytosis (3). Receptor endocytosis, in turn, is often coupled to ubiquitination (4–6). Once endocytosed, ubiquitinated receptors may be recycled, or they may be targeted via the ESCRT (endosomal sorting complexes required for transport) machinery for destruction in the lysosome (7). Thus multiple decision points determine whether the ultimate fate of activated receptors is rapid reactivation by dephosphorylation in situ, slower reactivation by recycling from endosomes to the plasma membrane, or degradation.

GPCR agonists and antagonists are among the largest single category of approved pharmaceuticals. The β2-adrenergic receptor in particular is a major drug target. Inhaled β2-adrenergic receptor agonists such as albuterol have been the primary treatment for asthma symptoms for decades. They continue to be used by millions of patients worldwide. In practice, the efficacy of these and other GPCR agonists as drugs is limited in part by the down-regulation of their receptors. The potential for the use of down-regulation inhibitors as potentiators of GPCR agonist potency and response duration is obvious. This study is motivated in part by the need to fill gaps in the basic structural mechanisms underlying β2-adrenergic receptor down-regulation.

The complex set of decisional nexa encountered by newly activated receptors highlight how critical the coupling to ubiquitin ligases is in this process. Although some transmembrane proteins, of which the epithelial sodium channel C-subunit (ENaC) is best known (8), have an intrinsic ability to couple to ubiquitin ligases, in most cases adaptor proteins are involved. The β and visual arrestin family has been intensively studied for more than two decades for its roles in GPCR desensitization (9) and has also been linked to GPCR ubiquitination (10). In the past five years it has become clear that a family of proteins containing arrestin homology domains (11), which includes six arrestin-related domain containing proteins (ARRDC1–5 and TXNIP) in humans and the arrestin-related transport proteins in yeast (12), has important roles in targeting Nedd4-family

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2The abbreviations used are: GPCR, G-protein-coupled receptor; ARRDC3, arrestin-related domain-containing protein-3; ENaC, epithelial sodium channel C-subunit.
ubiquitin ligases to receptors. These arrestin-related proteins seem in many cases to target their substrates for degradation. In particular, ARRDC3 has been proposed to have a major role in the down-regulation of the β2-adrenergic receptor in mice and in human cells (13, 14, 36), although contradictory observations have been reported (15).

The human proteome includes nine members of the Nedd4 ubiquitin ligase family (16). Nedd4 family members consist of an N-terminal membrane binding C2 domain, two to four WW domains with connecting linkers, and a C-terminal ubiquitin ligase HECT domain. The paradigmatic Nedd4 family reaction in human cells is the ubiquitination of the ENaC subunits by Nedd4-2 (8). Nedd4-2 binds to PPXY motifs at the C-termini of the subunits of ENaC via its WW domains.

WW domains consist of ~40 amino acid residues, which fold into a three-stranded antiparallel β-sheet. The WW domain is one of the most compact of all autonomously folded domains (17). WW domains are so-named for their two conserved tryptophan residues. WW domains are widespread in proteins involved in apoptosis, transcription, RNA splicing, and ubiquitination among others (18). Most WW domains bind Pro-containing peptide sequences, but the details of their specificity vary. WW domains have been divided into five groups based on their preference for binding different types of Pro-rich motifs (18). The four WW domains of Nedd4 belong to this group.

Solution NMR analyses of Nedd4 WW domain bound to PPXY peptides from ENaC (19, 20) provided the prototypical structural studies how Nedd4-family enzymes bind their substrates. Budding yeast contains just one Nedd4 ortholog, Rsp5. A recent structure of a large portion of Rsp5 bound to a PPXY-containing substrate, Sna3, showed how PPXY binding and ubiquitination are coordinated in three dimensions (21). Human ARRDC proteins and yeast arrestin-related transports contain PPXY motifs as one of their defining features. The natural hypothesis is that the ARRDCs bind to WW domains of Nedd4 ligases in the same way as substrates. However, no structural or quantitative biochemical data have been available to test this hypothesis or to understand the relative contributions of the various WW domains and PPXY motifs in this system. As part of a larger effort to understand how ARRDC3 and other ARRDCs might direct the ubiquitination and down-regulation of the β2-adrenergic receptor and other GPCRs, here we report a quantitative biochemical and atomic resolution structural dissection of the ARRDC3-Nedd4 interaction.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of ARRDC3 and Nedd4 Fragments**—All four WW domains of human Nedd4 and the WW2-3 and WW3-4 tandem constructs were subcloned into the pGST-parallel2 vector (22). These constructs span the following residues of Nedd4: WW1, 166–210; WW2, 346–386; WW3, 416–450; WW4, 467–500 (Fig. 1, A and B). The C-terminal tail fragment of ARRDC3 containing two PPXY motifs (Fig. 1, A and B) and spanning residues 341’–400’ (where the prime (’) denotes residues of ARRDC3) was cloned into pMBP-parallel2 (22). The WW domains and ARRDC3 C-terminal tail were expressed in *Escherichia coli* BL21-gold (DE3) cells (Agilent Technologies). After induction with 0.2 mM isopropyl β-D-
1-thiogalactopyranoside overnight at 16 °C, the cells were pelleted by centrifugation at 4000 × g for 10 min. Cell pellets were lysed by sonication in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM Tris(2-carboxyethyl)phosphine-HCl, and 1 mM PMSF. The lysate was centrifuged at 25,000 × g for 1 h at 4 °C. For WW domain preparations, the supernatant was bound to glutathione-Sepharose 4B resin (GE Healthcare). The eluted protein was further purified on a Hi Trap Q HP column. Peak fractions were pooled and digested with tobacco etch virus protease overnight at 4 °C. This material was incubated with nickel-nitrilotriacetic acid resin for 0.5 h to remove His,–tobacco etch virus protease. The sample was then applied to Superdex 75 16/60 column equilibrated with 150 mM NaCl, 25 mM Tris-HCl, pH 8.0.

For preparation of the ARRDC3 C-terminal tail construct, the supernatant was bound to nickel-nitrilotriacetic acid resin. The eluted protein was further purified on a Hi Trap Q HP column and a Superdex 200 16/60 column. The elution peaks were pooled and flash-frozen in liquid N2.

**Isothermal Titration Calorimetry**—Binding constants of the four WW domains of Nedd4–1 for the two PPXY motifs of ARRDC3 were measured using a MircoCal iTC200 system (GE Healthcare) at 25 °C. The purified WW domains were dialyzed overnight against 25 mM HEPES, pH 7.3, 150 mM NaCl. PPXY motif peptides (PPXY1, 341′–355′; PPXY2, 384′–399′) (New England Peptide) were dissolved into double-distilled water and adjusted to pH 7.0 with NaOH. The peptide solution was diluted to 1 mM using the WW domain dialysis buffer. 1.0 mM PPXY peptide solution was injected into a cell containing 0.1 mM WW domain. PPXY peptide samples were injected into dialysis buffer as a control. The curves were analyzed with Origin. Protein and peptide concentrations were determined by UV absorption across the 260–280-nm spectrum.

**Crystallography**—Nedd4–WW3 was concentrated to 20 mg/ml. Crystals were grown by hanging-drop vapor diffusion at 21 °C. To generate apoWW3 crystals, the WW3 solution was mixed with well buffer composed of 3.0 mM NaCl, 100 mM Tris-HCl, pH 8.0, at a 1:1 ratio. Crystals appeared with 24 h and grew to full size after 2 days. Crystals were flash-frozen with liquid N2 in a cryoprotectant solution of 20% glycerol, 0.35M (NH4)2SO4, 100 mM Tris-HCl, 3M NaCl, 100 mM Tris-HCl, pH 8.0. For crystallization of the WW3–PPXY1 complex, WW3 was mixed with PPXY1 peptide at a 1:3 molar ratio and incubated on ice for ~30 min. The complex crystals were grown in 0.35 M (NH4)2SO4, 100 mM Tris-HCl 8.0, and 100 mM guanidine-HCl. Complex crystals appeared and grew to full size within 24 h. The crystals were flash-frozen with liquid N2 in a cryoprotectant solution of 20% glycerol, 0.35 M (NH4)2SO4, 100 mM Tris-HCl 8.0, 100 mM guanidine-HCl.

Diffraction data were collected at the Advanced Photon Source (APS) beamline 22-ID. Data were processed with HKL2000 software (HKL Research). Data collection and processing statistics are given in Table 1. The WW3 apo structure was solved by a molecular replacement method using PDB coordinates 2HO2 A chain (human Fe65-WW domain as bound to a peptide from hMena (23)) as the search model. Molecular replacement was carried out with the program BALBES (24). The complex structure was solved by molecular replacement method with Phaser (25) using the WW3 apo structure as a search model. Model building and refinement were carried out with ccp4 (26), COOT (27), REFMAC5 (28), Phenix (29), and ARP/wARP (30).

**Immunoprecipitation**—The pCR3.1 YFP-ARRDC3 and p3 × FLAG CMV26 myc-Nedd4 plasmids were kindly provided by Dr. Martin-Serrano (King’s College) and Dr. Fadila Bouamr (National Institutes of Health), respectively. Mutations in the 3 × FLAG CMV26 myc Nedd4 plasmid were prepared by site-directed mutagenesis and confirmed by DNA sequence analysis (NIDCR shared resource facility). Mutations included W219A in the WW1 domain, W376A in the WW2 domain, W449A in the WW3 domain, and W501A in the WW4 domain, and combinations of two, three, or all four mutations. For immunoprecipitation, 1 μg of YFP-ARRDC3 and 1 μg of 3 × FLAG Nedd4 plasmid were co-transfected into HEK293 cells in 6-well plates using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were harvested with 300 μl of lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1% Triton X-100 and clarified for 10 min at 15,000 × g at 4 °C. A fraction of lysate (50 μl) was saved for input lysate, and the
remaining lysate was used for immunoprecipitation with anti-FLAG M2 affinity beads (Sigma) and rotated for 3 h at 4 °C. Beads were washed 3 times with Tris-buffered saline and eluted with sample buffer. Lysates were run on 10% SDS-PAGE gels for Western blot analysis. ARRDC3 antibody was obtained from Abcam (Cambridge, MA), FLAG antibody was purchased from Sigma, and all other antibodies were from Cell Signaling Technologies (Beverly, MA).

RESULTS

WW3 Has the Highest Affinity for PPXY Motifs of ARRDC3—To quantitatively determine how ARRDC3 recruits Nedd4, we performed isothermal titration calorimetry. Each of the four independent WW domains was prepared as a recombinant protein and purified. Peptides were synthesized corresponding to the two PPXY motifs of ARRDC3. The peptides PPXY1 and PPXY2 correspond to ARRDC3 residues 341–355 and 384–400, respectively.

The WW domains have affinities in the rank order WW3 > WW4 ~ WW2 >> WW1 (Fig. 2, A and B). WW3 binds to both PPXY1 and PPXY2 the most tightly of the four WW domains, with $K_d$ values of 3.3 ± 0.4 and 19 ± 3 μM, respectively. WW1 binds with both PPXY motifs weakly, with $K_d$ values that could not be quantitated accurately, but exceed 100 μM. WW2 and WW4 bind PPXY1 with intermediate affinity and bind PPXY2 weakly. A consistent trend is apparent in which PPXY1 binds severalfold more tightly than PPXY2 to all three of the WW domains where the affinities were high enough to measure.

Crystal Structure of the WW3-PPXY1 Complex—To understand the structural basis for the highest affinity interaction in the system, the Nedd4 WW3 domain was co-crystallized in complex with the PPXY1 peptide (Fig. 3). The key residues of the PPXY1 peptide are shown as sticks. Carbon atoms of the WW3 domain are colored dark green, and those of the PPXY1 peptide are colored yellow. Nitrogen is blue, and oxygen is red. An omit map of PPXY1 was generated at a contour level of 1σ (magenta).

| Table 1 Statistics of crystallographic data collection and refinement |
|-----------------------------|-----------------------------|
| WW3 apo                   | WW3-PPXY1 complex |
| Data collection            |                     |
| Space group                | P4,22               | P3,21               |
| Cell dimension             |                       |                      |
| $a$, $b$, $c$ (Å)          | 68.41, 68.41, 37.25   | 38.88, 38.88, 50.00 |
| $\alpha$, $\beta$, $\gamma$ (%) | 90, 90, 90        | 90, 90, 120         |
| Wavelength (Å)             | 1.00000             | 1.0000              |
| Resolution (Å)             | 50.00-1.10 (1.14-1.10) | 50.000-1.70 (1.76-1.70) |
| No. of reflections         | 36262               | 9331                |
| Completeness (%)           | 99.9 (98.9)         | 99.6 (98.0)         |
| Redundancy (%)             | 20.2 (6.9)          | 7.3 (6.0)           |
| $R$sym (%)                 | 8.1 (45.7)          | 6.5 (20.1)          |
| $I/\sigma(I)$              | 30.0 (4.4)          | 17 (8.7)            |
| Refinement                 |                     |                     |
| Resolution (Å)             | 32.73-1.1 (1.13-1.10) | 33.37-1.70 (2.14-1.69) |
| $R_{work}/R_{free}$ (%)    | 18.0/19.4 (19.4/20.8) | 19.3/22.6 (20.8/25.9) |
| Average $B$-factor (Å)     | Protein             | 24.5                | 28.7 |
|                            | Peptide             | 28.2                |      |
|                            | Root mean square deviation from ideality |                   |
| Bond length (Å)            | 0.008               | 0.005               |
| Bond angle (°)             | 1.4                 | 0.97                |
| Ramachandran Plot          | Favored (%)         | 100                 | 100 |
|                            | Allowed (%)         | 0                   | 0    |
|                            | Outlier (%)         | 0                   | 0    |
the PPX peptide, and the structure was determined at 1.7 Å resolution (Table 1, Fig. 3, A and B). The PPX peptide residues 344–348 are in a type II polyproline conformation (Fig. 4A), with ϕ angles ranging from −56 to −81 and ψ angles from 142 to 166 degrees. Residues 349–352 are in the 3_10-helical conformation. Residues 348 and 349 both participate in i to i+3 hydrogen bonds with residues 351 and 352, respectively (Fig. 4B). The peptide buries a total of 378 Å² surface area.

The type II polyproline conformation of the N-terminal region is stabilized by the packing of the two Pro of PPX peptide, Pro-346 and Pro-347, against Trp-449 and Phe-438, respectively (Fig. 4C). Two hydrogen bonds between WW3 side chains and the peptide backbone also contribute to binding and stabilization of this conformation (Fig. 4D). The first is formed between the carbonyl of Glu-344 of PPX and the side chain of Trp-449 of WW3. The second is formed between the carbonyl of Pro-347 of PPX and the side chain of Thr-447 of WW3. The third is between the side chains of Tyr-349 and His-342. E, Tyr-349 and Val-352 are snugly packed in a hydrophobic groove consisting of Arg-430, Ile-440, and Lys-445.

FIGURE 4. Structural interactions in the WW3-PPX complex. A, schematic model of the PPX motif showing the key Pro residues in the type II polyproline conformation. B, the intrapeptide hydrogen bonds that help stabilize the PPX conformation are show as magenta. C, Pro-346' and Pro-347' pack against Trp-449 and Phe-438, respectively. D, hydrogen bonds between WW3 and PPX. From right to left, the first is formed between the main chain carbonyl group of Glu-344' of PPX, and the side chain of Trp-449 of WW3. The second is formed between the carbonyl of Pro-347' of PPX, and the side chain of Thr-447 of WW3. The third is between the side chains of Tyr-349' and His-342. E, Tyr-349' and Val-352' are snugly packed in a hydrophobic groove consisting of Arg-430, Ile-440, and Lys-445.
hydrogen bond is formed between the carboxyl of Pro-347 and the side chain hydroxyl of Thr-447.

The $\beta_2$-$\beta_3$ turn (residues 442–445) in one of the two chains (A) is highly mobile. B-factors in this region of the A chain reach 200 Å², as compared with an average of 25 Å² for the entire structure. The main structural changes upon PPXY binding are as follows. The guanidine group of Arg-430 moves by 3 Å to make room for the side chain of Val-352’. The rotation of the side chains of Val-352’ packs more than 200 Å² for the entire structure.

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close contact between the Ile Cγ2 and the Thr Cγ atoms. The conformational change in the Thr side chain is very important for peptide binding because it contributes its hydroxyl group to donate a hydrogen bond to Pro-347 as described above. Thus an elegant set of coupled repacking interactions connects formation of the Val-352′ binding pocket for the C-terminal part of the peptide to formation of a key hydrogen bond with the N-terminal part of the peptide (Fig. 5C).

Basis for Affinity Differences between PPXY1 and PPXY2—To explore whether the tight packing of Val at the +3 position of PPXY1 contributes to its higher affinity as compared with PPXY2, where Val is replaced by Ile (Figs. 1 and 6A), a V352I peptide was prepared. The $K_d$ of WW3 domain and PPXY1 V352I was $8.7 \pm 0.8 \mu M$, a roughly 2-fold reduction compared with wild-type PPXY1 (Fig. 6B). The Val thus contributes to the high affinity but does not fully account for it.

Co-immunoprecipitations Are Robust to Mutation of Single WW Domains—A co-immunoprecipitation assay of YFP-ARRDC3 and FLAG-tagged Nedd4 demonstrated a robust interaction between these two proteins (Fig. 7A). Mutation of the WW3 domain (W449A) alone reduced association by roughly 2-fold. However, mutation of WW3 in combination with the WW2 (W376A) or WW4 (W501A) domains more significantly reduced the interaction with ARRDC3 (Fig. 7, A and B). Furthermore, mutation of the tryptophan residues of WW2, WW3, and WW4 (and of all four WW domains) completely abolished the co-immunoprecipitation, thus indicating the WW2, WW3, and WW4 domains of Nedd4 are required for interaction with ARRDC3.

Tandem WW Domains Have Very High Affinity for C-terminal Domain of ARRDC3—We sought to understand how the lower affinity interactions of the other three WW domains complement the high affinity binding of PPXY1 to WW3. Tandem constructs were generated that included the WW2-3 and WW3-4 pairs, and both PPXY motifs and their affinities were measured by isothermal titration calorimetry. These constructs bound with $K_d$ values of 510 and 300 nM, respectively (Fig. 8).

DISCUSSION

Our findings highlight the parallelism between the ARRDCs and PPXY-containing Nedd4 substrates. It appears that ARRDCs and arrestin-related transports likely evolved their Nedd4 family recruitment activity by recapitulating the same recognition principles used by Nedd4 substrates. As with the direct Nedd4 substrate ENaC (31–33), WW3 represents a focal point of affinity for ARRDC3. The WW3-PPXY1 complex resembles the ENaC/ENaC subunit complex (19) in the recognition of core PPXY residues. Indeed, these elements are shared in common by other group I WW-peptide complex structures (17, 21, 34).

The structural details that underpin the high affinity of the ARRDC3 PPXY1 interaction with WW3 appear on their surface to differ from the ENaC β subunit peptide complex. The PPXY motif of the ENaC β subunit forms what is described by the authors (19) as a single turn of α-helix C-terminal to the Tyr. The final residue of this single turn helix is a Leu-621′, three residues after the Tyr. The side chain of Leu-621′ contributes most of the additional buried surface area in the ENaC β subunit complex. The ARRDC3 PPXY1 motif also uses a C-terminal extension to bolster affinity. For the ARRDC3 motif, a 3₁₀ helix at the end of the ARRDC3 PPXY1 motif replaces the ENaC β subunit α-helix. Val-352′ of ARRDC3 binds to a similar unit complex.
hydrophobic surface region as Leu-621 of ENaC-

There are no other amino acid residue differences in surrounding regions of these two peptides that can readily explain the differences in the conformations. It seems worth considering that the correct structure of this portion of the ENaC-

The role of induced fit in Pro-rich motif recognition has been little discussed but was highlighted in a recent analysis of Nedd4 WW3 binding to ENaC-α subunit (35). This report found that the β2-β3 turn was highly dynamic in the absence of peptide binding. This observation is consistent with the disorder in the same turn in one of the two copies present in the crystallographic asymmetric unit. The present pair of bound and unbound structures illuminates additional details that would have been difficult to detect in structures at less than atomic resolution. We observed an elegant interconnection between hydrophobic repacking at one site with hydrogen bond formation at an adjacent site. Repacking around the key Val-352' side chain forces a change in rotamer for Thr-447. This is, in turn, essential for Thr side-chain hydrogen bonding to the type II polyproline segment. This previously unappreciated induced fit mechanism may contribute to the specificity of the WW3 for PPXY motifs with a hydrophobic residue three positions C-terminal to the Tyr.

The finding that PPXY₁ has the highest affinity for Nedd4 WW domains in vitro is consistent with the finding that PPXY₁ is also the more important of the two PPXY motifs for Nedd4 recruitment (13). Our observation that the interaction between Nedd4 and ARRDC3 persists to at least some extent in the WW3-inactivated mutant is consistent with the previous finding that the interaction survives deletion of either PPXY motif individually (13). This interaction seems to be important enough that it has been “over-engineered,” at least when using overexpressed proteins as done in our experimental conditions and those of Nabhan et al. (13).

Nevertheless, the interaction between Nedd4 and ARRDC3 could be inhibited by disrupting multiple PPXY-WW domain interactions. Because Nedd4 contributes to the ubiquitination and down-modulation of β2-adrenergic receptor, blocking Nedd4-ARRDC3 interactions could potentially provide an opportunity for potentiating and prolonging β2-adrenergic
receptor signaling, which is particularly relevant for effective treatment of asthma.

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