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Protein Domain-Domain Interactions and Requirements for the Negative Regulation of *Arabidopsis* CDC48/p97 by the Plant Ubiquitin Regulatory X (UBX) Domain-containing Protein, PUX1*

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CDC48/p97 is an essential AAA-ATPase chaperone that functions in numerous diverse cellular activities through its interaction with specific adapter proteins. The ubiquitin regulatory X (UBX)-containing protein, PUX1, functions to regulate the hexameric structure and ATPase activity of AtCDC48. To characterize the biochemical mechanism of PUX1 action on AtCDC48, we have defined domains of both PUX1 and AtCDC48 that are critical for interaction and oligomer disassembly. Binding of PUX1 to AtCDC48 was mediated through a region containing both the UBX domain and the immediate C-terminal flanking amino acids (UBX-C). Like other UBX domains, the primary binding site for the UBX-C of PUX1 is the N_a domain of AtCDC48. Alternative plant PUX protein UBX domains also bind AtCDC48 through the N terminus but were found not to be able to substitute for the action imparted by the UBX-C of PUX1 in hexamer disassembly, suggesting unique features for the UBX-C of PUX1. We propose that the PUX1 UBX-C domain modulates a second binding site on AtCDC48 required for the N-terminal domain of PUX1 to interact with and promote dissociation of the AtCDC48 hexamer. Utilizing Atcdc48 ATP hydrolysis and binding mutants, we demonstrate that PUX1 binding was not affected but that hexamer disassembly was significantly influenced by the ATP status of AtCDC48. ATPase activity in both the D1 and the D2 domains was critical for PUX1-mediated AtCDC48 hexamer disassembly. Together these results provide new mechanistic insight into how the hexameric status and ATPase activity of AtCDC48 are modulated.

CDC48 and its mammalian ortholog, p97/valosin-containing protein (VCP),[2] are highly abundant and conserved members of the AAA family (ATPases associated with diverse cellular activities) of molecular chaperones (for a review, see Refs. 1–5). The active form of CDC48/p97 is a ring-shaped complex comprised of six identical ~90-kDa subunits containing three domains: an N-terminal domain followed by two Walker-type ATPase domains (D1 and D2) that are joined by conserved linker regions.

CDC48/p97 protomers have the intrinsic ability to self-oligomerize, thereby creating the active hexameric ATPase (6–8). Recent studies have revealed that the D2 domain of CDC48/p97 is responsible for the major enzyme activity of the chaperone (9, 10). In contrast to the D1 domain, mutations in the D2 domain that inhibit ATP hydrolysis or binding displayed dominant negative properties *in vivo*. Ultrastructural studies of p97 have suggested that CDC48/p97 nucleotide hydrolysis and exchange in the D2 ATPase domain result in conformational changes that drive movement of the N-terminal domain. This provides the mechanical force necessary to act on substrate proteins (7, 11–13).

Targeting of CDC48/p97 activity to cellular pathways is accomplished via its recruitment by adapter/cofactor proteins. For example, p97 requires the cofactor p47 to modulate endoplasmic reticulum/Golgi membrane assembly (14–16) and nuclear envelope reformation (17). Alternatively, the cofactor heterodimer complex Ufd1-Npl4 is required for p97-mediated formation of the chromatin-associated nuclear envelope network, consolidation of the nuclear envelope (17), spindle dynamics (18), and endoplasmic reticulum-associated protein degradation (19–21). The interaction of studied adapter/cofactor proteins with CDC48/p97 occurs via the ~200 amino acid N-terminal domain of CDC48/p97 (11, 13, 22, 23). The N-terminal domain of CDC48/p97 is also responsible for multiubiquitin chain binding necessary for the *in vitro* degradation of target proteins (24, 25). However, binding of p47 and Ufd1-Npl4 to p97 is mutually exclusive (26). Recent studies have also shown that proteins interact via the C terminus of CDC48/p97 (27–29).[3]

The CDC48/p97 adapter p47 contains two independent CDC48/p97 binding sites, the ubiquitin regulatory X (UBX) domain and binding site 1 (BS1) (11, 30). The two binding sites interact independently with CDC48/p97. The UBX domain is comprised of ~80 amino acids and shares common secondary structure organization with ubiquitin, characterized by a β-β-

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[2] The abbreviations used are: VCP, valosin-containing protein; UBX, ubiquitin regulatory X; PUX, plant UBX-containing protein; aa, amino acids; GST, glutathione *S*-transferase; TEV, tobacco etch virus; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; VAT, VCP-like ATPase.

[3] D. M. Rancour and S. Y. Bednarek, manuscript in preparation.

TABLE 1
Oligonucleotides used in this study

Name	Sequence (5′-3′)	Purpose
SB42	CATATTCATTGATCAGATCGACTCTATTGCACCG	5′ Site-directed mutagenesis for Atcdc48 E308Q
SB43	CGGTGCAATAGAGTCGATCTGATCAATGAATATG	3′ Site-directed mutagenesis for Atcdc48 E308Q
SB44	CCATGTGTTCTTTTCTTTGATCAGCTCGACTCC	5′ Site-directed mutagenesis for Atcc48 E581Q
SB45	GGAGTCGAGCTGATCAAAGAAAAGAACACATGG	3′ Site-directed mutagenesis for Atcdc48 E581Q
SB371	tcagtatcaGCGGCCGCAGTAATCCAAAAGTAGAG	3′ H6T7-AtCDC48; NotI
SB439	ggaattcCATATGCATCATCATCATCATCAC	5′ H6T7-AtCDC48; NdeI
SB524	ctGGATCCTCGTTGAAGACA	5′ 13 amino acid extension from UBX of PUX1; BamHI
SB525	ttGGATCCGCAGATTTTTACCG	5′ 27 amino acid extension from UBX of PUX1; BamHI
SB526	gGAATTCTCAACTACCACCATCATCT	3′ 10 amino acid extension from UBX of PUX1; EcoRI
SB527	gGAATTCTCATTTGGTCATGG	3′ 30 amino acid extension from UBX of PUX1; EcoRI
SB606	TTATGGACCACCTGGGTCTGGAGCGACTTTGATC	5′ Site-directed mutagenesis for Atcdc48 K254A
SB607	GATCAAAGTCGCTCCAGACCCAGGTGGTCCATAA	3′ Site-directed mutagenesis for Atcdc48 K254A
SB608	CTTTTCTACGGTCCTCCTGGATGTGGGGCAACCTT	5′ Site-directed mutagenesis for Atcdc48 K527A
SB609	AAGGTTGCCCCACATCCAGGAGGACCGTAGAAAAG	3′ Site-directed mutagenesis for Atcdc48 K527A
SB680	CCCGGGAAACAGATGGCTC	5′ H6T7-Atcdc48 (214–809); SmaI
SB697	TTACCTGACACCACCAACATCATC	3′ H6T7-Atcdc48 (1–213)
SB814	CTCGAGCTAGAATTTCTGGATGACGACGG	3′ PUX5 UBX (335–421); XhoI
SB815	GAATCCATCAAGAGGGCTAGTTGTAGA	5′PUX5 UBX (335–421); EcoRI
SB820	TTAGTACTTGACGTCTGGGCATTGGT	3′ H6T7-Atcdc48 (1–114)
SB821	CCCGGGGGAAAGCGTGTTCACAT	5′ H6T7-Atcdc48 (115–213); SmaI
SB823	CTACCTGACACCACCAACATCATCATAA	3′ H6T7-Atcdc48 (115–213)
SB824	CCCGGGCCTGTGAAGAGAGAGGAT	5′ H6T7-Atcdc48 (192–809); SmaI

α-β-β-α-β β-Grasp fold. In contrast to ubiquitin, UBX domains lack the C-terminal glycine motif necessary for covalent coupling to target proteins and are typically located toward the C terminus of a variety of eukaryotic proteins that interact with CDC48/p97 (14, 31). *Saccharomyces cerevisiae* encodes seven UBX-containing proteins that have been shown to interact with Cdc48p. Deletion of the UBX domains abolishes their binding to Cdc48p, indicating that the UBX domain is a general Cdc48p binding module (32). In addition to the UBX domain, p47 contains a second CDC48/p97 interaction domain known as BS1, which is located between the C-terminal UBX domain and a conserved SEP domain of undefined function. The hydrophobic residues of BS1 in p47 are conserved in Ufd1 (located between residues 215 and 241) and are believed to be involved in the exclusive nature of interaction of CDC48/p97 with either p47 or Ufd1-Npl4 (11).

The *Arabidopsis* genome encodes a family of 15 UBX-containing proteins.[4] Recently we have shown that one of these proteins, PUX1, can regulate the oligomeric status and activity of the *Arabidopsis* CDC48/p97 ortholog, AtCDC48, as well as mammalian p97 but not the related AAA ATPase *N*-ethylmaleimide-sensitive factor (33). PUX1 association with the CDC48/p97complex inactivated the ATPase and promoted the disassembly of the hexamer *in vitro*. Loss-of-function *pux1* mutants displayed accelerated growth relative to wild-type plants, suggesting that PUX1 functions as a negative regulator of AtCDC48 function *in vivo*.

Here we have defined further the interaction regions between PUX1 and CDC48/p97 and the requirement of the D1 and D2 ATPase domains for PUX1-mediated hexamer disassembly. Our results have demonstrated that the central UBX domain of PUX1 interacts through the N terminus of AtCDC48. In addition, we showed that the nucleotide status of AtCDC48 influences PUX1-mediated disassembly of the hexameric ATPase.

[4] D. M. Rancour and S. Y. Bednarek, unpublished data.

EXPERIMENTAL PROCEDURES

Oligonucleotides Used in This Study—All oligonucleotides used in this study were synthesized by Integrated DNA Technologies (Coralville, IA) (Table 1). Capitalized sequences represent those complementary to the *PUX1*, *PUX5*, or *AtCDC48*. Lowercase nucleotides correspond to sequences added to aid in cloning. Restriction enzyme and point mutant sequences are underlined.

Expression and Purification of GST-free and GST-tagged PUX Fusion Proteins—GST-tagged full-length PUX1, UBX-C terminus (amino acids (aa) 101–251), and the N-terminal domain (aa 1–100) of PUX1 were prepared as described (33). All other pux1 truncation mutant constructs used in this study were generated by PCR using primers listed in Table 1 and subcloned into the bacterial expression vector pGEX4T-3-TEV (33). The PUX5 (At4g15410) cDNA clone C104907 (34) was obtained from *Arabidopsis* Biological Resource Center (Ohio State University) and verified by DNA sequencing. The PUX5 cDNA was used as a template for PCR amplification with primers SB814/815 (Table 1) of the region encoding the UBX domain (aa 335–421). The amplified product was cloned into pGEM®-T easy (Promega) verified by DNA sequencing and subcloned as an EcoRI fragment into the bacterial expression plasmid pGEX4T-2-TEV (33). Protein expression was performed in the *ROSETTA* (Novagen) *Escherichia coli* strain. Cultures were grown at 37 °C to mid-log phase and induced with 250 (PUX5 constructs) or 100 μM (PUX1 constructs) isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were disrupted by sonication in cold Tris-buffered saline, pH 7.4, 1 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 1 mM *p*-aminobenzamidine, 1 mM ϵ-aminocaproic acid, 5 μg/ml aprotinin, and 1 μg/ml leupeptin and affinity-purified using glutathione-Sepharose 4B as described (35). GST-free proteins were prepared by cleavage with His_6-tagged TEV protease. All *E. coli* expressed proteins were quantified by the Bradford method using bovine serum albumin as a standard, and their purity was determined by SDS-PAGE and Coomassie Blue staining.

Cloning and E. coli Protein Expression of H6T7-tagged AtCDC48 and Atcdc48 Mutant Proteins—Full-length H6T7-AtCDC48 containing an N-terminal tag consisting of 6 histidine residues followed by the 12 amino acid T7 epitope tags (H6T7) was generated as described (33) and used for the production of H6T7-tagged Atcdc48 mutant proteins. Truncation mutant constructs used in this study were amplified by PCR from the H6T7-AtCDC48 cDNA using primers listed in Table 1. Single and double ATP binding and hydrolysis mutants of H6T7-Atcdc48 D1 (K254A and E308Q) and D2 (K527A and E581Q) were also engineered. Site-directed mutagenesis by the Stratagene QuikChange protocol (see Table 1 for oligonucleotide sequence details) was used to alter residues that have previously been shown to be required for CDC48/p97 ATPase activity (25, 36–38). For the binding mutants, the lysine residue in the D1 and D2 Walker A motif was replaced with alanine. For the hydrolysis mutants, the glutamate residue of the Walker B DE*XX* motif was changed to glutamine (see Fig. 2*A*, *constructs 6–11*). Mutant sequences were verified by DNA sequencing and subcloned into the bacterial expression vector pET29A (Novagen). Wild-type and mutant H6T7-AtCDC48 proteins were expressed in the *ROSETTA* (Novagen) strain of *E. coli* at 28 °C using 100 μM isopropyl-1-thio-β-D-galactopyranoside for 2 h and purified using nickel-nitrilotriacetic acid (Qiagen Inc., Valencia, CA) as described (33).

In Vitro Binding Studies—PUX1 binding assays contained 1 μg of purified *E. coli*-expressed H6T7-AtCDC48 or H6T7-Atcdc48 mutant proteins in 100 μl of reaction buffer (20 mM HEPES/KOH, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.1% (v/v) Triton X-100). Full-length and GST-pux1 truncation mutant proteins were added at a molar ratio of 3:1 relative to H6T7-AtCDC48 or H6T7-Atcdc48 mutant proteins. Reactions were incubated in reaction buffer for 30 min on ice followed by affinity isolation using glutathione-Sepharose 4B resin (Amersham Biosciences). Isolated complexes were washed five times with binding buffer and subjected to SDS-PAGE followed by either staining with Coomassie Brilliant Blue (R-250) or immunoblotting with anti-T7 antibodies (Novagen).

ATPase Enzyme Activities of Atcdc48 Mutant Proteins—Kinetic analysis was performed as described previously (33) with purified *E. coli*-expressed wild-type and mutant H6T7-Atcdc48. Assays were conducted in ATPase reaction buffer (20 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol) with the specified ATP concentrations (0.05–0.2 mM) at 22 °C. Assay time parameters were empirically determined for all wild-type and mutant H6T7-Atcdc48 proteins to ensure that the initial rates of catalytic activity in the single time point assays were measured to ensure a fit to a Michaelis-Menten model. Colorimetric detection of phosphate product release was performed in a 96-well microtiter plate format, and absorbance measurements were measured using a microplate reader (Bio-Tek, Winooski, VT) equipped with a 660-nm filter. Data analysis and curve fitting were performed using GraphPad Prism version 4 (GraphPad Software, Inc, San Diego, CA). Activity values represent the ± S.D. of triplicate determinations.

Analysis of Oligomeric Structure of Full-length and Mutant Atcdc48/pux1 Complexes—To assess the oligomeric status of full-length and mutant H6T7-Atcdc48 and the effect of PUX1-derived proteins on their structure, PUX1-derived proteins were mixed with wild-type and mutant H6T7-Atcdc48 proteins at a molar ratio of 3:1 and incubated on ice for 30 min prior to sucrose gradient fractionation. Approximately 20 μg of total protein was fractionated on a 5-ml 20–40% (w/w) sucrose gradient (20 mM HEPES/KOH, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol). Protein sedimentation standards were analyzed on parallel gradients. Gradients were fractionated as described (33), and the fractions were subjected to SDS-PAGE followed by immunoblotting with anti-T7 antibodies. Refractive indices of gradient fractions were used to allow comparison of different gradients.

RESULTS

The PUX1 UBX Domain Interacts with AtCDC48—Recent studies have suggested that the UBX motif may serve as a general CDC48/p97 interaction domain (11, 20, 32, 39, 40). Consistent with this, we have shown that a fragment of the PUX1 protein containing a UBX domain with an additional 70 C-terminal amino acids (UBX-C terminus; aa 101–252; Fig. 1*A*) binds to AtCDC48 (33). The PUX1 UBX-C terminus together with the N-terminal domain (aa 1–100) of PUX1 can mediate AtCDC48 complex disassembly *in trans*. When compared with the majority of UBX-containing proteins, the predicted PUX1 UBX domain, however, has several atypical features. First, the PUX1 UBX domain is positioned near the middle of the protein (Fig. 1*A*) as opposed to the C terminus observed in most other proteins. Second, the PUX1 UBX domain lacks a conserved phenylalanine-proline di-amino acid motif in the proposed protein loop between the S3 and S4 β-strands that is postulated to be required for the interaction of the p47 UBX domain with the N terminus of CDC48/p97 (11, 40).

To test whether the isolated PUX1 UBX domain is sufficient for binding to AtCDC48, we examined the interaction of a GST-tagged fusion protein containing the UBX domain but lacking the C-terminal 70 amino acids with AtCDC48 *in vitro*. Based upon the SMART (Simple Modular Architecture Research Tool) protein motif search algorithm, the PUX1 UBX domain resides between amino acids 101 and 181. Unfortunately, a GST fusion protein containing this 81-amino-acid region was insoluble when expressed in *E. coli* (data not shown). The addition of the 13 and 30 amino acids that flank the N and C terminus of the "SMART-defined" UBX domain, respectively, was required to maintain the solubility of a GST-tagged UBX fusion protein (GST-UBX aa 88–211). As shown in Fig. 1*B*, GST-UBX (aa 88–211) bound to AtCDC48 with approximately the same efficiency as full-length PUX1 protein. In contrast, binding to AtCDC48 of a soluble truncated GST-UBX fusion protein, GST-UBX (aa 88–191), lacking the 19 C-terminal amino acid segment (aa 192–211), was nearly abolished (Fig. 1*B*).

Similar to the PUX1 UBX-C terminus truncation mutant (aa 101–252) (33), the GST-free UBX (aa 88–211) region greatly promoted the binding (Fig. 1*C*, *lane 5*) of the PUX1 N-terminal domain (aa 1–100) to AtCDC48 and subsequent dissociation of

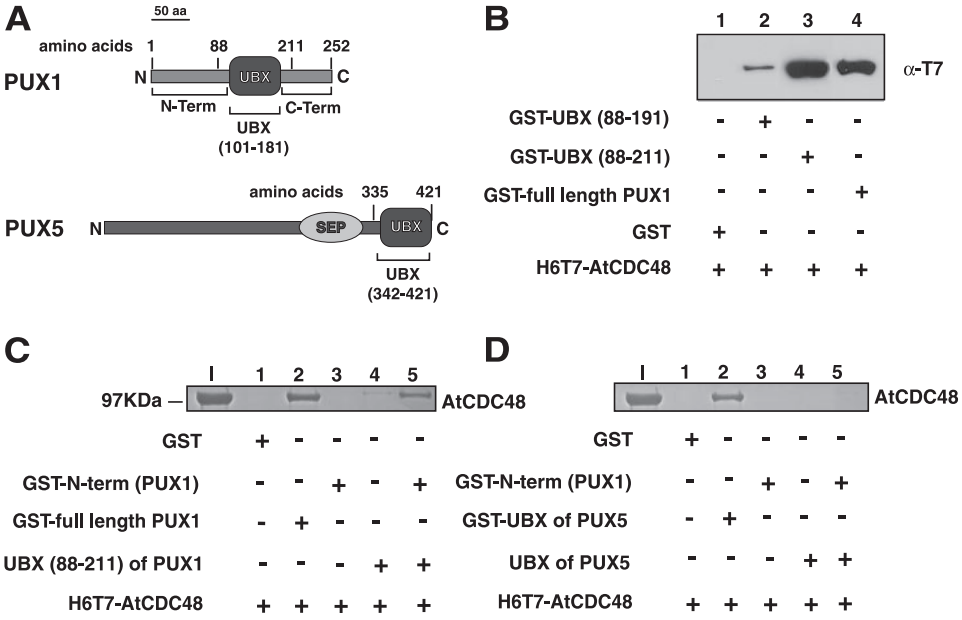

FIGURE 1. **The UBX (aa 88–211) domain of PUX1 is required for interaction with AtCDC48.** *A*, protein domain organization of *Arabidopsis* PUX1 and PUX5 (At4g15410). *N-Term*, N terminus; *C-Term*, C terminus. *B*, *E. coli*-expressed GST-UBX truncation fusion proteins of PUX1 (*lanes 2* and *3*) or full-length GST fusion of PUX1 (*lane 4*) were incubated with H6T7-AtCDC48 protein at a 3-to-1 molar ratio for 30 min at 4 °C, isolated with glutathione-Sepharose 4B resin, and analyzed by immunoblotting with anti-T7 antibody. *C*, PUX1-N terminus binds AtCDC48 in the presence of the PUX1 UBX (aa 88–211). GST or GST fusion protein of PUX1 was incubated with H6T7-AtCDC48. Protein complexes were isolated with glutathione-Sepharose 4B, washed, and analyzed by SDS-PAGE and Coomassie Blue staining. *D*, PUX5 UBX binds AtCDC48 but does not promote PUX1 N terminus binding. GST or GST fusion protein of PUX1 was incubated with H6T7-AtCDC48. Protein complexes were isolated with glutathione-Sepharose 4B, washed, and analyzed by SDS-PAGE and Coomassie Blue staining. *I*; input.

the AtCDC48 hexamer *in trans* (data not shown). In the absence of GST-free UBX (aa 88–211), we did not detect any interaction between AtCDC48 and the PUX1 N-terminal domain (Fig. 1*C*, *lane 3*), similar to previous observations (33).

To determine whether all UBX domains could support the *in trans* PUX1 N terminus domain association with AtCDC48 or whether it was a unique feature of the PUX1 UBX, we examined whether the UBX domain from another AtCDC48-interacting PUX protein could also mediate the interaction of the N terminus of PUX1 with AtCDC48. For these experiments, the UBX domains of PUX5 (At4g15410) and PUX4 (At4g04210) were chosen to be analyzed due to their high sequence similarity to mammalian p47. PUX5, PUX4, and PUX3 (At4g22150) represent the closest *Arabidopsis* homologues to mammalian p47.[4] As shown in Fig. 1*D* (*lane 2*), a GST fusion protein containing the PUX5 UBX domain (aa 332–421) (Fig. 1*A*) bound to AtCDC48. In contrast to PUX1 UBX (aa 88–211), GST-free PUX5 UBX (aa 332–421) did not promote binding of the PUX1 N-terminal domain (Fig. 1*D*, *lane 5*), although it bound to AtCDC48 with similar efficiency as GST-PUX5 (data not shown). Similar results were obtained with the UBX domain from PUX4 (data not shown). These data suggest that a functional difference exists between UBX domains for CDC48/p97 complex disassembly.

Domain Interactions between AtCDC48 and PUX1—Previous studies have shown that the UBX domain of p47 and several other UBX domain-containing proteins bind to the N-terminal domain of CDC48/p97 protomers (11, 20, 21, 32, 39, 40). Given the difference we observed between PUX1 and PUX5 UBX domain-mediated binding of the PUX1 N terminus, we wanted to define the region(s) of AtCDC48 required for PUX1 interaction. For this purpose, we generated several AtCDC48 truncation mutants (Fig. 2*A* and *B*, *constructs 2–5*) and tested their ability to bind GST-PUX1 *in vitro* (Fig. 3*A*). Binding of PUX1 to the N-terminal deletion mutant (aa 192–809) was not detectable (Fig. 3*A*), although the mutant was competent to assemble into an ATPase-active oligomeric complex (Figs. 3*B* and 4 and Table 2). Velocity sedimentation analysis also demonstrated that PUX1 was unable to mediate disassembly of the AtCDC48 N-terminal (aa 192–809) deletion mutant (Fig. 3*B*). As shown in Fig. 3*A*, PUX1 was found to bind to a fusion protein containing the N-terminal 213 amino acids of AtCDC48, suggesting that the N terminus of AtCDC48 is the main interacting domain of PUX1.

To define further which subdomain(s) of the N terminus of AtCDC48 are required for interaction with PUX1, GST-tagged fusion proteins containing the N-terminal AtCDC48 subdomains, N_a (aa 1–114), and N_b (aa 115–213) were generated based on previous studies (41). *In vitro* binding studies demonstrated that the N_a (aa 1–114) region of AtCDC48 contains the critical binding site(s) for PUX1 (Fig. 3*A*). These data support a model whereby the PUX1 UBX domain associates primarily through the N_a-terminal domain of AtCDC48 to mediate complex disassembly.

Role of ATP Binding and Hydrolysis in PUX1-mediated AtCDC48 Interaction and Dissociation—To determine whether the ability of PUX1 to bind and consequently disassemble oligomeric AtCDC48 is dependent upon the ATPase activity of the D1 and/or D2 domains of the chaperone, we generated a series of AtCDC48 truncation and point mutants (Fig. 2) that putatively affect the function of the D1 and D2 ATPase domains.

E. coli-expressed Atcdc48 mutant proteins (Fig. 2*A*, *constructs 6–11*) were soluble, and their purity was confirmed by SDS-PAGE (Fig. 2*B*, *lanes 6–11*). To further characterize these Atcdc48 mutant proteins, we assessed their ability to oligomerize and to hydrolyze ATP.

Previously, we have shown that *E. coli*-expressed wild-type AtCDC48 has robust ATP hydrolytic activity. The enzyme exhibited a K_m of 40.5 μM and a V_{max} of 12.5 μM P_i min^{-1} μM^{-1} (33). Assuming that each hexamer has six active sites (25, 37), a K_{cat} of 2.1 mM P_i min^{-1} active $site^{-1}$ was determined for wild-type AtCDC48. Several AtCDC48 mutant proteins exhibited ATPase activity that fit a Michaelis-Menten steady-state kinetic model including Atcdc48 (aa 192–809) and, surpris-

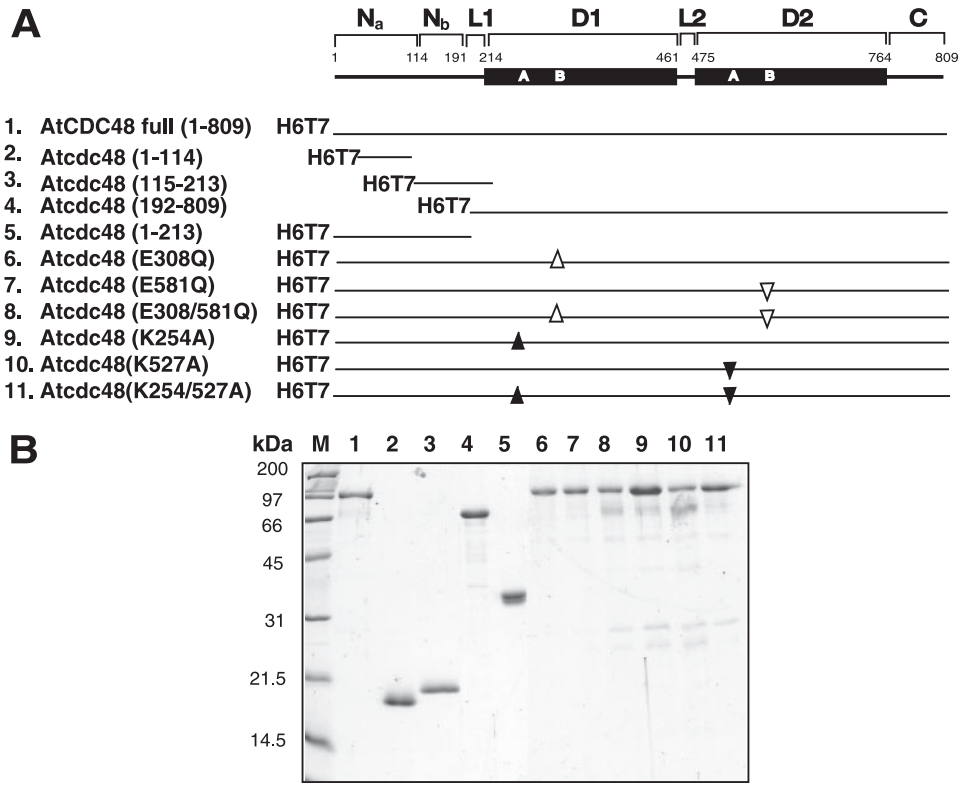

FIGURE 2. **AtCDC48 proteins used in this study.** *A*, schematic representation of AtCDC48 wild-type and mutant proteins. The site-specific mutations in the ATPase domains are depicted by △ (D1) ▽ (D2) for ATP hydrolysis and ▲ (D1) ▼ (D2) for ATP binding mutants. *N*, N-terminal domain; *L*, linker regions; *C*, C-terminal domain. *B*, purified wild-type and mutant H6T7-Atcdc48 proteins. One microgram of H6T7-tagged fusion proteins was resolved by SDS-PAGE and stained with Coomassie Blue. The molecular mass markers are shown on the left (*M*), and *lane numbers* shown above correspond to order of wild-type (*1*) mutants (*2–11*) shown in *panel A*.

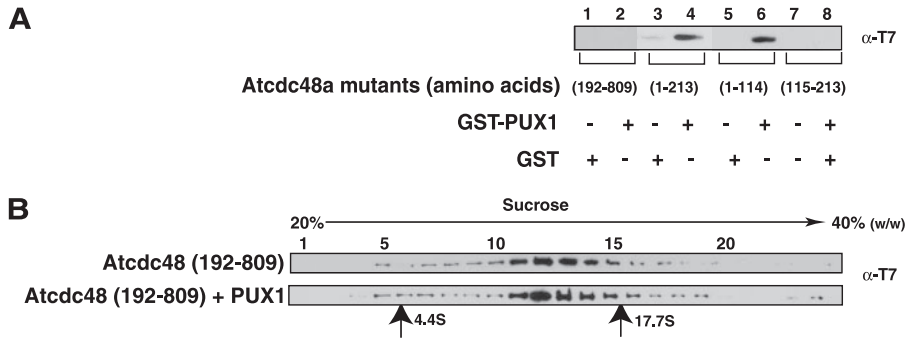

FIGURE 3. **The N_a subdomain of the AtCDC48 N terminus (aa 1–114) is the primary binding site for PUX1.** *A*, *E. coli*-expressed GST-fused PUX1 and H6T7-Atcdc48 truncation protein binding assays. Atcdc48 truncation mutant proteins were incubated with either GST-fused PUX1 (*lanes 2*, *4*, *6*, and *8*) or GST alone (*lanes 1*, *3*, *5*, and *7*) for 30 min at 4 °C. Protein complexes were isolated with glutathione-Sepharose 4B. Bound protein was washed extensively and analyzed by SDS-PAGE followed by immunoblotting for H6T7-Atcdc48 using anti-T7 antibody. *B*, *E. coli*-expressed GST-free PUX1 and H6T7-tagged Atcdc48 (aa 192–809) were incubated together at a 3-to-1 molar ratio on ice for 30 min in the absence of exogenous nucleotide and fractionated by sucrose gradient (20–40%) velocity sedimentation. Gradient fractions were analyzed by SDS-PAGE followed by immunoblotting. The migration of sedimentation marker proteins bovine serum albumin (4.4 *s*, 66 kDa) and apoferritin (17.7 *s*, 480 kDa) is indicated. Protein peak migrations were identical between gradients as determined by the refractive indices of the gradient fractions.

ingly, D1 ATP hydrolysis and binding point mutants, Atcdc48 (E308Q) and Atcdc48 (K254A), respectively (Table 2). A modest ATP hydrolytic activity was detected for Atcdc48 D2-deletion mutant (aa 1–475), but data could not be fit to a Michaelis-Menten steady-state kinetic model. D2 ATP hydrolysis (E581Q), D2 ATP binding (K527A) mutants, and both D1/D2 ATP hydrolysis (E308Q/E581Q) and binding (K254A/K527A) double mutants did not exhibit any detectable ATPase activity. While performing our enzyme kinetic analysis, we observed that the L1 containing Atcdc48 (aa 192–809) mutant exhibited a K_{cat} of 2.9 mM P_i min^{-1} active $site^{-1}$, which is 1.4 times the wild-type value (Table 2). In addition, this truncation mutant exhibited cooperativity in its ATPase cycle manifest in a Hill coefficient of 2 (Fig. 4), suggesting a negative effect of the N-terminal domain on ATP turnover rates for AtCDC48.

All Atcdc48 mutant proteins whose activity was tested were subjected to gradient analysis to determine whether they formed oligomers. All proteins listed in Table 2 oligomerized except for Atcdc48 (aa 214–809). The Atcdc48 truncation mutant (aa 214–809) lacking both the N-terminal domain as well as the 22-amino-acid linker region, L1, showed no detectable ATPase activity and did not properly assemble into a hexameric complex (data not shown), suggesting a critical role for the L1 in oligomerization of AtCDC48.

To determine whether ATP binding and/or hydrolysis influence the interaction of PUX1 to AtCDC48, wild-type and mutant H6T7-Atcdc48 were incubated with GST-PUX1 in the presence and absence of ATP and affinity-purified using immobilized glutathione. As shown in Fig. 5*A*, PUX1 interaction with AtCDC48 was independent of the nucleotide status of the hexameric complex. In addition, the PUX1 N-terminal domain was able to bind H6T7-Atcdc48 double hydrolysis and binding mutants in the presence of the PUX1 UBX-C (Fig. 5*B*). Therefore, binding of PUX1 to AtCDC48 is not dependent on the ATP status nor the capacity of AtCDC48 to bind or hydrolyze ATP.

Although binding of PUX1 to AtCDC48 is not dependent on ATP, we examined whether PUX1-mediated disassembly of AtCDC48 was influenced by the ATPase activity of AtCDC48. We first verified by velocity sedimentation analysis that all single and double mutants assembled into oligomeric complexes with sedimentation characteristics indistinguishable from

wild-type *E. coli*-expressed H6T7-AtCDC48 (Fig. 6) (33). We then tested whether PUX1 could promote the disassembly of these mutant forms of AtCDC48. As shown in Fig. 6, *A* and *B*, PUX1 facilitated dissociation of the single D1 or D2 ATP hydrolysis (E308Q and E581Q) and ATP binding (K254A and K527A) mutant oligomeric H6T7-Atcdc48 complexes. PUX1-mediated disassembly of hexameric D1/D2 double ATP hydrolysis and binding mutants (Fig. 6, *A* and *B*), however, was significantly inhibited.

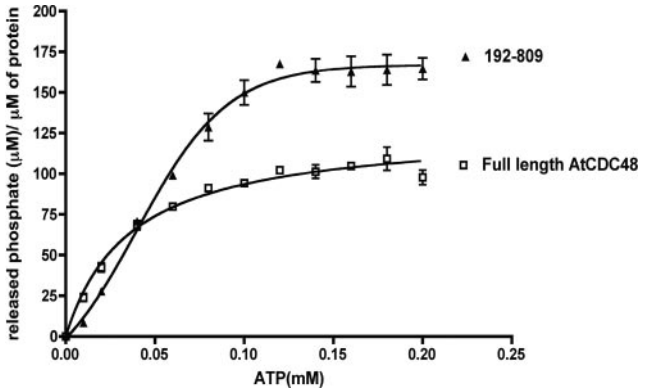

FIGURE 4. **ATPase activity of AtCDC48 and Atcdc48 mutant (aa 192–809).** The substrate concentration dependence of ATPase activity for the full-length protein and aa 192–809 is shown. A 10-min fixed point ATPase assay was used. Values represent the mean ± S.D. of three independent measurements.

TABLE 2

Steady-state enzyme kinetics of H6T7-Atcdc48 mutants

−, no detectable activity.

Mutation	K_m (μM)	V_{max} (μM P_i min^{-1} μM^{-1})	K_{cat} (μM P_i min^{-1} active $sites^{-1}$)[a]	Normalized ATPase activity
Wild type	40.5	12.5	2.1	1
192–809	100	17.2	2.9	1.4
214–809	–	–	–	–
1–461	–	–	–	–
1–475[b]	16	4.1	0.7	ND
E308Q	50	8.8	1.5	0.7
E581Q	–	–	–	–
E308Q/E581Q	–	–	–	–
K254A	57	3.5	0.6	0.3
K527A	–	–	–	–
K254A/K527A	–	–	–	–

[a] K_{cat} calculated as the hexamer of six active sites.
[b] The mutant exhibited minimal activity that could not be fit with Michaelis-Menten kinetics therefore the normalized ATPase activity was not determined (ND).

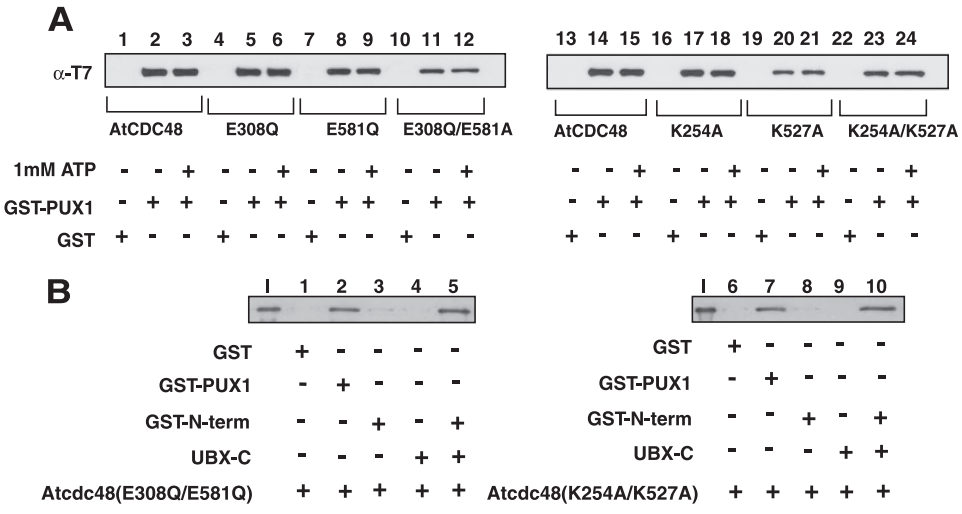

FIGURE 5. **Binding of PUX1 and AtCDC48 is ATP independent.** *A*, *E. coli*-expressed GST-PUX1 and H6T7-Atcdc48 ATPase mutant protein binding assays. Purified GST (*lanes 1*, *4*, *7*, *10*, *13*, *16*, *19*, and *22*) or GST-PUX1 was incubated with Atcdc48 mutant proteins in the presence (*lanes 2*, *5*, *8*, *11*, *14*, *17*, *20*, and *23*) or absence (*lanes 3*, *6*, *9*, *12*, *15*, *18*, *21*, and *24*) of 1 mM ATP for 30 min at 4 °C. *B*, *in vitro* binding assays were performed using GST-fused N terminus of PUX1 with either Atcdc48 (E308Q/E581Q) (*lanes 1–5*) and Atcdc48 (K254A/K527A) (*lanes 6–10*). Protein complexes were isolated and washed, and bound protein was analyzed by SDS-PAGE followed by immunoblotting for Atcdc48 using anti-T7 antibody. *I*, input.

DISCUSSION

Plant UBX-containing protein 1, PUX1, was identified through protein interaction studies and found to be a regulator of AtCDC48 function *in vitro* and *in vivo* (33). This work provided direct evidence that the hexameric structure of the CDC48/p97 family member is under dynamic protein-mediated regulation. In this study, we focused in greater detail on the structural and nucleotide requirements for PUX1 interaction with AtCDC48 and their role in the AtCDC48 disassembly process. This work has identified several distinct features of AtCDC48 that regulate hexamer formation and influence its ATPase activity. These are likely to be common to the regulation of other CDC48/p97 family members.

Previous work has demonstrated the capacity of CDC48/p97 orthologs to bind ubiquitin and ubiquitin-like protein domains of the β-GRASP fold family (14, 31). This protein-protein interaction feature is used to facilitate the recruitment of CDC48/p97 to a variety of biological processes either via adapters that contain these protein folds or by direct binding to ubiquitin. Our previous work with PUX1 established the requirement of a domain region that included the PUX1 UBX-C terminus region (aa 101–252) in binding AtCDC48. Further deletion analysis of PUX1 (Fig. 1*A*) narrowed the domain requirements of PUX1 for binding to AtCDC48 to PUX1 amino acids 88–211, which included the UBX domain and 30 amino acids of the C-terminal flanking sequence. Inclusion of these 30 additional C-terminal flanking amino acids had significant consequences for binding, but it is unclear whether this is due to direct binding of the flanking amino acids to AtCDC48 or their requirement for truncated protein stability. These issues remain to be tested.

CDC48/p97 interaction with ubiquitin and ubiquitin-like protein folds of the β-GRASP fold family have been shown to be mediated through the N-terminal domain of CDC48/97. Similarly, we show that the primary binding interaction of PUX1 with AtCDC48 occurs through the N terminus of AtCDC48 (Fig. 3*A*). Truncation mutants of AtCDC48 lacking the N-terminal domain (aa 192–809) lose their ability to bind PUX1. The N-terminal domain of CDC48/p97 has been further divided into two subdomains, referred to as N_a and N_b, based on structural analysis of CDC48/p97 (41). Analogous truncation mutants of AtCDC48 were

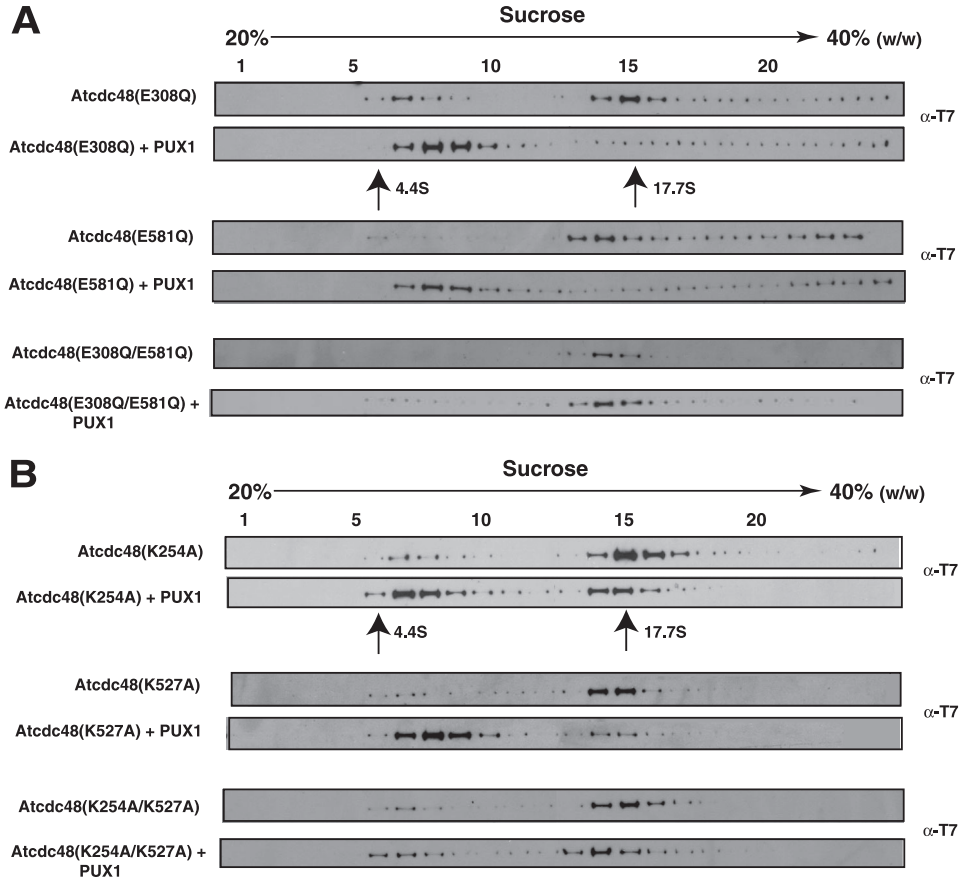

FIGURE 6. **ATP hydrolysis and binding in both D1 and D2 ATPase domain is required for PUX1-mediated AtCDC48 disassembly.** *A*, Atcdc48 double ATP hydrolysis mutant is resistant to PUX1-mediated disassembly. *B*, Atcdc48 double ATP binding mutant is resistant to PUX1-mediated disassembly. Sucrose gradient sedimentation analysis of Atcdc48 site-directed mutant proteins in the absence or presence of PUX1 was performed. *E. coli*-expressed GST-free PUX1 (26.8 μg) and H6T7-tagged Atcdc48 mutant proteins (20 μg) were mixed in a 3-to-1 ratio, incubated 30 min on ice in the absence of exogenous nucleotide and fractionated by sucrose gradient (20–40% (w/w)) velocity sedimentation. The migration of sedimentation marker proteins bovine serum albumin (4.4 *s*, 66 kDa) and apoferritin (17.7 *s*, 480 kDa) is indicated.

made, and protein binding studies indicated that the N_a region from AtCDC48 (aa 1–114) was necessary and sufficient for binding of PUX1 UBX-containing regions (Fig. 3*A*). These data, taken together, suggest that PUX1 has evolved to take advantage of the conserved binding site for β-GRASP folds to bind and mediate hexamer disassembly. Binding of the PUX1 UBX domain to AtCDC48 is mutually exclusive with the UBX domain of PUX4, which displays amino acid sequence similarity to mammalian p47 (data not shown).

In addition to the N_a and N_b regions, our data also indicated that the linker 1 between N terminus and D1 domain of AtCDC48 (aa 192–213) was critical for hexamer stability and ATPase activity of AtCDC48. Without linker 1, the N terminus deletion mutant of Atcdc48 did not form an oligomer and thus lost its ATPase activity.

It has been reported that the conformation of the N terminus of CDC48/p97 changes upon nucleotide binding and hydrolysis (7, 12, 13, 42). In addition to the binding of interacting proteins, the N-terminal domain of AtCDC48 regulates the ATPase activity of AtCDC48. An Atcdc48 lacking its N-terminal domain (retaining aa 192–809) showed cooperative ATP binding and higher ATPase activity than wild type (Fig. 4). Similarly, the N-terminal deletion of the *Thermoplasma* VCP-like ATPase (VAT) showed up to 24-fold enhanced ATP hydrolysis and 250-fold enhanced protein unfolding activity when compared with wild-type VAT, indicating that N terminus either regulates and/or inhibits the ATPase activity of VAT (43). Our hypothesis is that the N terminus of AtCDC48 is important for negatively regulating ATP hydrolysis in the absence of protein substrates.

In addition to the structural requirements necessary for PUX1 binding, we also showed that PUX1 interaction with AtCDC48 was independent of ATP hydrolysis and binding in the D1 or D2 ATPase domains (Fig. 5*A*). Oligomerization of full-length *E. coli*-expressed AtCDC48 is not affected by the exclusion of exogenous nucleotide nor by the presence of ATP, ADP, and AMP-PNP (33). Likewise, the Walker A/Walker B mutant Atcdc48 proteins generated in this study assembled into hexamers, providing additional evidence that hexamerization of AtCDC48 is independent of the nucleotide hydrolysis and/or binding capacity of the ATPase domains (Fig. 6).

Previously, we showed that PUX1-mediated AtCDC48 hexamer disassembly is only partially inhibited in the presence of the ATP analog, AMP-PNP (33). In this work, we have directly investigated the individual and combined contributions of the ATPase domains for PUX1-mediated AtCDC48 disassembly. PUX1 was found to mediate disassembly of single Walker A/Walker B ATP hydrolysis and binding Atcdc48 mutant proteins (Fig. 6). However, the double Walker A and B mutants exhibited significant inhibition in their ability to go through the disassembly process than either of the single ATPase domain mutants. We hypothesize that inhibition of both the D1 and the D2 ATPase domains alters the conformational flexibility of AtCDC48, preventing the necessary conformational changes required for hexamer disassembly. These conformational changes are apparent due to the ability of the PUX1 N terminus to bind in the absence of the UBX-C (Fig. 5*B*, *lanes 3* and *8*), but the mechanistic downstream steps for disassembly do not occur, nor have they been resolved. Unfortunately, attempts at mapping the binding site of the PUX1 N terminus have not been successful to date.

The overall structure of the CDC48/p97 hexamer is drastically influenced by the ability of the two ATPase domains to bind and subsequently hydrolyze ATP (7, 12, 13). These dynamic structural changes are postulated to be the primary mechanism through which the CDC48/p97 family of ATPases functions as

molecular chaperones. This plasticity in structure appears to be taken advantage of by PUX1, resulting in the demise of AtCDC48 through disassembly of the active hexamer.

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