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Ubiquitination of the tomato cell death suppressor Adi3 by the RING E3 ubiquitin ligase AdBiL

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ABSTRACT

Programmed cell death (PCD) is an organized process by which organisms selectively remove cells according to developmental needs or in response to biotic or abiotic stress. Despite recent efforts to understand mechanisms by which cell death takes place in plants, several gaps remain in our understanding of the molecular elements involved. The tomato PCD suppressor Adi3 is an AGC kinase that shares functional homology with the mammalian inhibitor of apoptosis PKB. Regulation of PKB stability, cell localization, and activation state is achieved through post-translational modifications such as ubiquitination. In an effort to understand the regulation of Adi3 function, we studied its interaction with the E3 ubiquitin ligase AdBiL. Using *in vitro* ubiquitination assays we show that AdBiL is an active E3 ubiquitin ligase using the E2 ubiquitin ligase UBC8 to ubiquitinate Adi3. Adi3 is also degraded in a proteasome-dependent manner. Our data draws additional parallels between Adi3 and PKB to support the functional relationship between these two PCD regulators.

1. Introduction

Ubiquitination is an eukaryotic post-translational modification by which ubiquitin (Ub), a 76 residue polypeptide, is covalently attached to a Lys residue in a target protein [1]. Ubiquitination has been linked to processes as diverse as protein degradation, endocytosis, histone modification, protein activation, responses to stress, DNA damage repair, and changes in intracellular protein localization [2]. Ubiquitination is accomplished through the coordinated action of Ub activating E1 (a.k.a. UBA) and conjugating E2 (a.k.a. UBC) enzymes and a Ub E3 ligase, which mediates transfer of ubiquitin to substrate and may or may not be a temporary carrier for the Ub to be transferred [3].

Arabidopsis thaliana contains two E1 enzymes, 37 proteins containing the characteristic E2 ligase UBC domain, and approximately 1300 E3s [1]. The different types of E3s have been grouped into four categories according to mechanism of action and subunit composition. E3 ligases containing RING and U-box domains mediate Ub transfer to substrates by bringing together E2s and substrates via a zinc-binding finger [4]. The HECT ligases are the only E3 ligases that harbor a cysteine residue to which activated Ub is loaded prior to transfer to substrates [5]. Finally, cullin-RING ligases contain a RING domain mediating E2 interaction, but posses a multi-subunit recognition system substrates interaction [3].

Evidence suggests ubiquitination plays a role in regulating programmed cell death (PCD) [6], the active process that leads to the removal of cells that are no longer required, have been damaged, or are infected [7]. In plants, PCD plays a fundamental role in gametogenesis, tracheary element development, senescence, and pathogen infection [7,8]. Several E3 ligases appear to be involved in PCD activation, particularly in response to pathogens [9–11]. However, the mechanisms by which these ligases mediate plant PCD are poorly characterized.

The tomato (*Solanum lycopersicum*) Ser/Thr protein kinase Adi3 is a suppressor of cell death involved in the resistance response to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* [12–14]. Adi3 is functionally similar to the mammalian cell death suppressor protein kinase B (PKB, a.k.a. Akt) [15]. Both Adi3 and PKB are activated by phosphorylation of an activation loop Ser by 3-phosphoinositide-dependent kinase 1 (PDK1) [12,15]. Loss of function of Adi3 and PKB causes the spontaneous appearance of cell death lesions, whereas their overexpression can prevent the development of PCD [12,15]. Furthermore, both kinases act as inhibitors of SnRK (AMPK in mammals); a master metabolism regulating complex [14,16]. In an effort to better understand the role of Adi3 in PCD regulation, we recently characterized interacting proteins through a yeast two hybrid (Y2H) screen [14,17]. One of these proteins, AdBiL (Adi3 Binding E3 Ligase)is a RING E3 ligase. Here we show that AdBiL posses E3 ubiquitin ligase activity and that it ubiquitinates Adi3 *in vitro*.

Abbreviations: AdBiL, Adi3 Binding E3 Ligase; PCD, programmed cell death; PKB, protein kinase B; RING, really interesting new gene; Ub, ubiquitin; UBA, ubiquitin activating enzyme; UBC, ubiquitin conjugating enzyme.

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2. Materials and methods

2.1. Cloning of tomato and Arabidopsis ubiquitination enzymes

Primers used in this study can be found in Supplemental Table 1. The open reading frame (ORF) of AdBiL was obtained by RT-PCR using leaf total RNA and corresponds to the Sol Genomics Network (SGN) unigene U580180. Full length ORF cDNAs were obtained from The *Arabidopsis* Information Resource (TAIR) clones: the E1 ligase *At*UBA1 (At2g30110) from clone U218214, *At*UBC8 (At5G41700) from the clone U15399, and *At*UBC11 (At3G08690) from the clone U18004. Tomato *Sl*UBC8 (SGN-U578242/U312900) was isolated from the EST clone cTOA-26-L24. *Sl*UBC10 (SGN U312900/U581187) was isolated from the EST clone cTOF-12-E20.

2.2. Recombinant protein expression and purification

MBP- and GST-tagged ubiquitination enzymes were obtained by expressing their ORF in the expression vectors pMAL-c2 (New England Biolabs) and pGEX-4T (GE-Healthcare). Proteins were expressed and purified as previously described [12,14]. For pull down assays, C-terminal FLAG-tagged AdBiL was obtained by cloning the AdBiL ORF into the pFLAG-CTC expression vector (Sigma–Aldrich).

2.3. Yeast two-hybrid

Y2H assays where conducted using the LexA expression vectors pEG202 for the bait constructs and pJG4-5 for the prey constructs as described previously [12,14].

2.4. α-Adi3 antibody design

Rabbit polyclonal antibodies were raised against an Adi3 synthetic peptide corresponding to residues 465–479 (IRIS-SDDPSKRGAAF) conjugated to the keyhole limpet hemocyanin (Covance Research Products, Denver, PA). Antibodies were tested using recombinant protein and an Adi3 antibody titer of 1:1000 to 1:500.

2.5. In vitro pull down assays

Pull down assays were carried out as previously described [20], except AdBiL-FLAG was purified in buffer supplemented with 0.2% Sarkosyl, 1 mM PMSF, and 1% Triton X-100, and wash buffer contained 10 mM Tris pH8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100.

2.6. Ubiquitination assays

In vitro ubiquitination assays were done as described [18] with the following modifications. The ubiquitination buffer used contained 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 5 mM ATP, 5 μg of FLAG tagged ubiquitin (Boston Biochem), 50 ng of purified E1, 250 ng of E2s, 400 ng of AdBiL and 1 μg of MBP-Adi3 or 3 μg of GST-Adi3 for pull downs. Proteins were separated by 8% SDS–PAGE and western blotting was used to identify ubiquitinated proteins using α-FLAG (Sigma) at 1:1000, α-MBP at 1:5000, α-GST at 1:5000, and α-Adi3 at 1:500. Human E2s were obtained from the UbcH Enzyme Set (Boston Biochemical). For pull down experiments, ubiquitination reactions were bound for 1 h at 4 °C to glutathione beads pre-equilibrated in binding buffer (50 mM Tris pH7.5, 0.5 mM DTT, 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100), washed six times in binding buffer containing 500 mM NaCl and pulled down proteins analyzed by 8% SDS PAGE and western blotting as described above.

2.7. Cell-free degradation assays

Cell free degradation assays were done as described [19,20] using 10 μg of soluble protein from three-week old tomato leaves in the presence of 500 ng of purified MBP-Adi3 or MBP-Adi3^{K337Q}. Reactions were incubated at 30 °C for 30, 90, or 180 min and stopped with addition of SDS–PAGE sample buffer. Proteins were resolved by 10% SDS–PAGE and analyzed with western blotting using α-Adi3 (at 1:500) and α-MBP (at 1:5000) antibodies.

2.8. Kinase assay

Kinase assays were done as previously described [12].

3. Results

3.1. Adi3 interacts with AdBiL

A yeast two hybrid (Y2H) screen with a cDNA library obtained from *P. syringae* pv. *tomato* infected tomato plants was used to identify Adi3 interacting partners [14,17]. The incomplete ORF of an RING domain containing protein obtained in the Y2H screen and was named AdBiL AdBiL (Adi3 Binding E3 Ligase). Using BLAST searches against the SGN database (http://solgenomics.net/), unigene U580180 was found to contain an AdBiL full length ORF. This sequence was used as a template to design primers to amplify the complete AdBiL ORF using RT-PCR on leaf total RNA. Surprisingly, no additional related E3 ligases were found in the tomato genome suggesting no redundant AdBiL sequences. Using the *Arabidopsis* information resource (TAIR) databases, the closest sequence homolog to AdBiL was found to be the protein encoded by At3g05545 (Supplemental Fig. S1; similarity: 58.5%, identity: 46.6%) followed by the red and far-red insensitive 2 protein (RFI2 – At2g47700; similarity 39.9%, identity 29.5%; Supplemental Fig. S1).

The full-length AdBiL ORF was used to confirm the Y2H interaction with Adi3 (Fig. 1A and B). The *Drosophila melanogaster* transcription factors BICOID and DORSAL were used as controls for false positive interactions. AdBiL did not auto-activate when expressed either in the prey or bait vectors or in the presence or absence of BICOID or DORSAL (Fig. 1A and B). An interaction was observed only when AdBiL was expressed (as either bait or prey) in the presence of Adi3 (Fig. 1A). This interaction was found to be independent of the Adi3 kinase activity since AdBiL still interacted with kinase-inactive (K337Q) or constitutively-active (S539D) Adi3 mutants (Fig. 1B). These interactions were tested in an *in vitro* immunoprecipitation experiment. FLAG-AdBiL was expressed in *E. coli*, protein extracts incubated in the presence or absence of MBP-tagged Adi3, and the complexes immunoprecipitated with an α-MBP antibody. AdBiL was co-immunoprecipitated only in the presence of Adi3 and, in accordance to Y2H results (Fig. 1B), was found to be unaffected by the kinase activity state of Adi3 (Fig. 1C). Collectively, this data suggest Adi3 can interact with AdBiL and this interaction does not rely on the kinase activity of Adi3. In support of these observations, an *in vitro* kinase assay revealed Adi3 does not, or only marginally *trans*-phosphorylates AdBiL (Supplemental Fig. S2).

3.2. AdBiL is an E3 ubiquitin ligase

AdBiL contains a characteristic RING finger domain, which includes an octet of Cys and His that bind zinc. RING domains can either have two His (RING-H2: C3H2C3) or one His (RING-HC: C3HC4) residue [3]. The AdBiL motif corresponds to a RING-H2

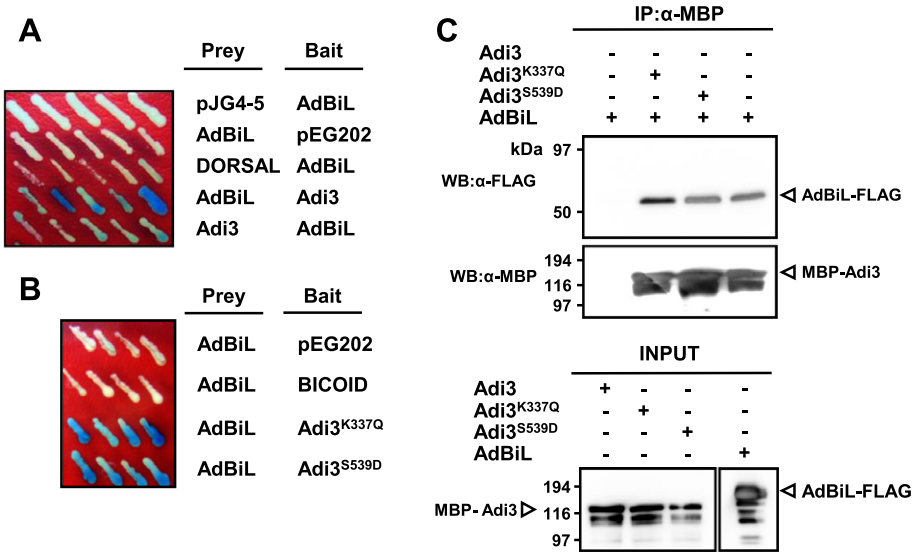

Fig. 1. Adi3 interaction with AdBiL. AdBiL interacts with Adi3 (A) and Adi3 kinase activity mutants (B) in a Y2H assay. Interaction was estimated by the expression of *LacZ* on plates containing X-Gal (blue = interaction). (C) Adi3 interacts with AdBiL *in vitro*. MBP and FLAG tagged proteins were bound to amylose resin for one hour at 4 °C. Co-immunoprecipitated proteins were analyzed by Western blotting with α-MBP and α-FLAG antibodies. Bottom panels, input for each fusion protein was analyzed by α-MBP and α-FLAG Western blot.

domain (Supplemental Fig. S1). RING domains occur in several protein families and they function in mediating protein–protein or protein-DNA interactions [3,4,21]. Because the closely related *Arabidopsis* AdBiL sequence At3g05545 has been shown to behave as an E3 Ub ligase that auto-ubiquitinates in the presence of *Arabidopsis* UBCs *At*UBC8, *At*UBC11, and to a lesser extent *At*UBC10 [21,22], we examined if AdBiL behaves as an E3 ligase using *in vitro* ubiquitination assays with MBP-tagged *At*UBA1 as the E1 enzyme and a set of human E2 UBCs. The transfer of FLAG tagged Ub to AdBiL was used as an indicator of reaction ubiquitination efficiency. Western blotting using an α-FLAG antibody revealed that human UBCs were incapable of transferring Ub to AdBiL (Fig. 2, top panel, lanes 3–8). Two different possibilities for this result were considered. First, it is possible that the *At*UBA1 (E1) is not compatible with the human E2s for loading Ub on these human E2s. The reciprocal situation however does not hold true, as multiple studies have successfully used human and yeast UBAs in order to activate plant UBCs [21]. Second, the human UBCs tested might be unable to interact with and transfer Ub to AdBiL.

In order to overcome these possible situations, *Arabidopsis* and tomato UBCs were used instead. Recombinant GST-tagged *At*UBC8 and *At*UBC11 were produced and used in *in vitro* ubiquitination assays. *At*UBC8, but not *At*UBC11 transferred Ub onto AdBiL as observed by the appearance of multiple bands in the α-FLAG western blot (Fig. 2, top panel, lanes 9 and 10). These bands indicate the transfer of one or multiple Ub moieties onto AdBiL. To the best of our knowledge, no previous characterization of tomato UBCs has been done, so BLAST searches against the SGN databases were used to identify tomato UBCs related to *At*UBC8 and *At*UBC11 using, not only these sequences, but closely related UBCs members of the *Arabidopsis* group VI of E2 UBCs [21]. From this search, seven UBC-related sequences were identified. The first five were named *Sl*UBCa–e and did not have noticeable sequence identity to *Arabidopsis* UBC proteins (Supplemental Fig. 3). The final two sequences were named *Sl*UBC8 and *Sl*UBC10 based on sequence similarity to and phylogenetic clustering with *At*UBC8 and *At*UBC10 (Supplemental Fig. 3). The tomato UBC8 and UBC10 were isolated from cDNA clones in the SGN EST library and tested for the ability to transfer Ub to AdBiL. Out of the two, only *Sl*UBC8 was capable of transferring Ub to AdBiL (Fig. 2, top panel, lane 11). Altogether these data indicate, AdBiL is specifically ubiquitinated in the presence of *At*UBC8 or *Sl*UBC8.

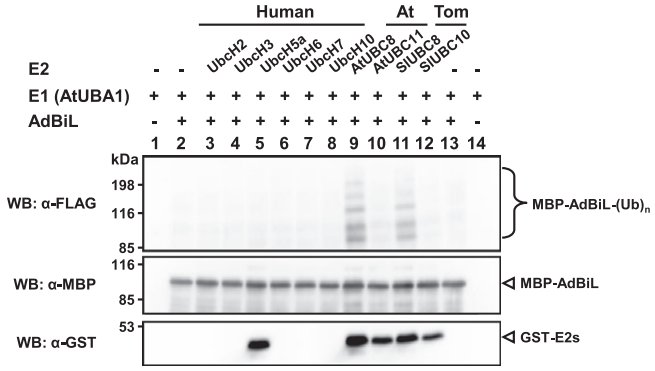

Fig. 2. AdBiL is ubiquitinated *in vitro*. Human, *Arabidopsis*, and tomato E2s were screened for their ability to transfer FLAG-tagged ubiquitin to MBP-tagged AdBiL in ubiquitination assays using the *Arabidopsis* E1 *At*UBA1. Ubiquitination reactions were separated by SDS–PAGE and Western blotting was conducted using α-FLAG to identify ubiquitinated proteins and α-MBP or α-GST antibodies to confirm assay inputs.

3.3. Production of an Adi3 antibody

An Adi3-specific antibody was developed to aid the *in vitro* and *in vivo* studies of Adi3. An Adi3 peptide (residues 465–479) was synthesized and used to develop polyclonal antibodies (Supplemental Fig. 4). Pre- and post-immunization serum was tested for recognition of recombinant Adi3 in western blots (Supplemental Fig. 4). Next, antibodies were affinity purified using the immobilized immunization peptide, and tested in western blotting with decreasing amounts of Adi3 (Supplemental Fig. 4B). The antibody immunoreacted with Adi3. However, the titer was too low to detect endogenous Adi3 in plant leaf extracts (data not shown) and thus, was used only for *in vitro* experiments.

3.4. AdBiL ubiquitinates Adi3

In order to determine if AdBiL mediates Ub transfer to Adi3, *in vitro* ubiquitination assays were carried out using *Sl*UBC8 as

the E2 enzyme. As seen previously, *Sl*UBC8 was capable of ubiquitinating AdBiL (Fig. 3A, top panel, lane 4). The addition of Adi3 to the ubiquitination reaction caused a drastic increase in the Ub signal when only E1, E2, and AdBiL were present (Fig. 3A, top panel, compare lanes 4 and 11). This would suggest Adi3 is in fact ubiquitinated by AdBiL. However, since the background generated by ubiquitination of AdBiL made it difficult to positively determine if Adi3 was being ubiquitinated, we used a GST pull down assay to selectively isolate GST-Adi3 from the ubiquitination assay. This assay showed that Ub-FLAG was detected in several distinct Adi3 bands suggesting Adi3 ubiquitination by AdBiL (Fig. 3B, top panel, lane 8). The presence of a few bands and a lack of a high molecular weight smear suggests Adi3 is not poly-ubiquitinated, but instead incorporates a few Ub moieties (Fig. 3B, top panel, lane 8). It should also be noted that detection of Adi3 using the α-Adi3 antibody in the full Ub assay showed a decrease of non-ubiquitinated Adi3 (Fig. 3B, middle panel, lane 8) suggesting a shift of some Adi3 to the Ub form. Also, ubiquitinated Adi3 was not detected with the α-Adi3 antibody presumably because of low amounts of ubiquitinated Adi3 and low titer of this antibody. Interestingly, the same ubiquitinated Adi3 bands were detected at a low level when only the E1, E2, or E3 were in the assay (Fig. 3B, top panel, lanes 1–6) suggesting a low level of ubiquitin transfer to Adi3 without the full enzyme complex.

3.5. Adi3 is de graded in a cell-free system

Possibly the best characterized outcome of ubiquitination is degradation by the 26S proteasome [23]. To explore this possibility for Adi3 ubiquitination, we used a cell-free system to test recombinant Adi3 stability in tomato leaf extracts in the absence or presence of increasing concentrations (0–100 μM) of the reversible proteasomal inhibitor MG132 [20]. MBP-tagged Adi3 was incubated with tomato leaf extracts and protein degradation was estimated by western blotting with the α-Adi3 or α-MBP antibodies (Fig. 4A). Adi3 was rapidly degraded in the presence of the tomato leaf extract without MG132 and virtually no protein remained after 1.5 h (Fig. 4A, lanes 1–4). Interestingly, the degradation of Adi3 was accompanied by the appearance of two cleavage products; one that immunoreacted with the α-Adi3 antibody at approximately 60 kDa (Fig. 4A, top panel) and one recognized by the α-MBP antibody at approximately 47 kDa (Fig. 4A, middle panel). Including increasing amounts of MG132 partially protected Adi3 from being completely degraded after 3 h of incubation, indicating at least partial involvement of the 26S proteasome in Adi3 degradation. The level of Adi3 after MG132 treatment did not reach that at the start of the assay most likely because MG132 is a reversible inhibitor of the proteasome and does not offer full protection against proteasomal degradation [24].

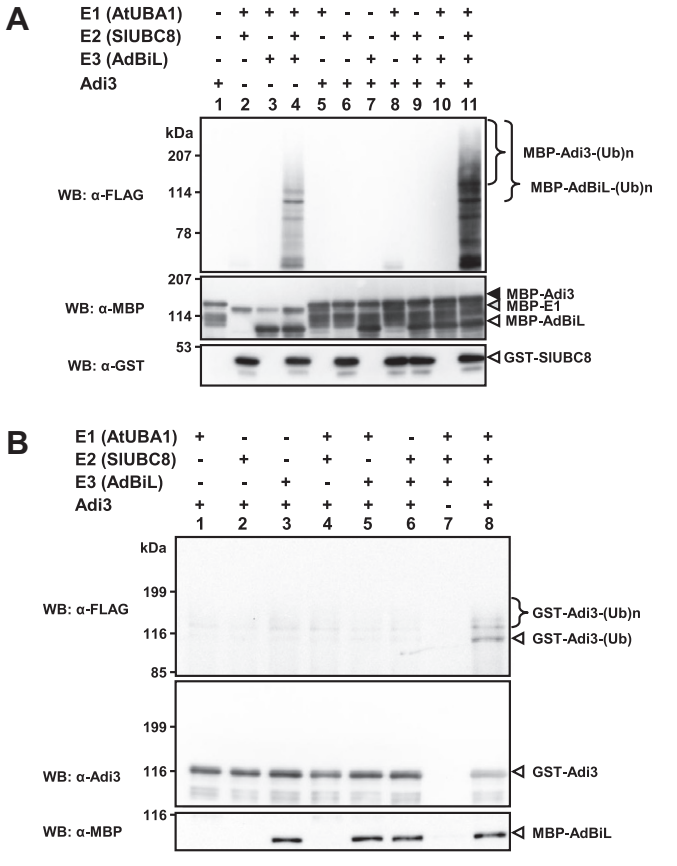

Fig. 3. Pull down of Adi3 ubiquitinated by AdBiL. (A) *In vitro* ubiquitination assay using MBP-*At*UBA1, GST-*Sl*UBC8, and MBP-AdBiL as the E1, E2, and E3 enzymes, respectively. MBP-Adi3 was used as the final substrate for the ubiquitination reaction. (B) *In vitro* ubiquitinated GST-Adi3 was precipitated using glutathione resin and resolved by SDS–PAGE. Western blotting was conducted using α-FLAG to identify ubiquitinated Adi3 (upper panel), α-Adi3 was used to detect the position of MBP-Adi3 in the blot (middle panel). The residual MBP-AdBiL bound to Adi3 was detected with α-MBP antibodies (lower panel).

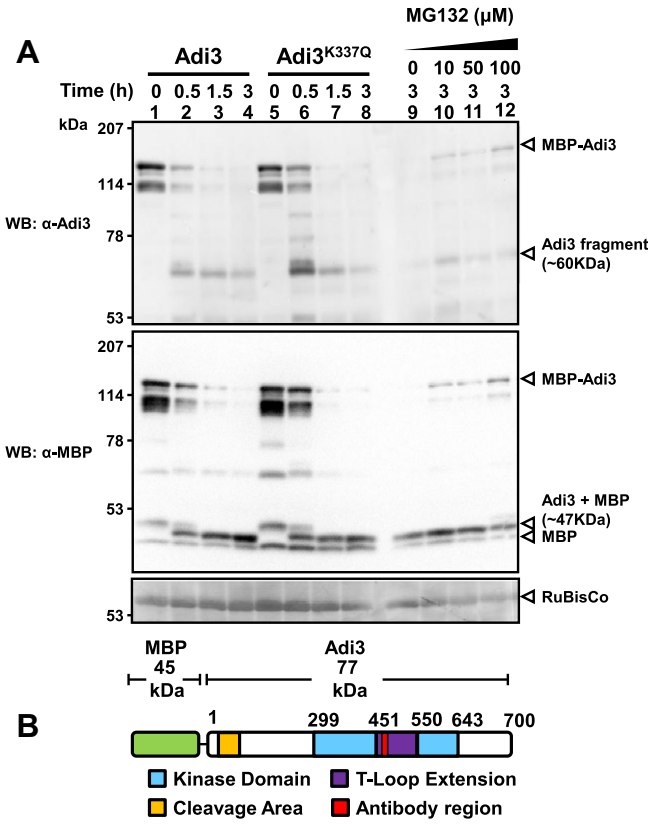

Fig. 4. Cell-free degradation of Adi3. (A) Recombinant Adi3 and kinase-inactive Adi3^{K337Q} are degraded in a proteasomal-dependent manner. MBP-Adi3 was incubated with tomato leaf protein extracts (10 μg) for the indicated times at 30 °C in the absence or presence of increasing MG132 concentrations. Adi3 levels were estimated by Western blotting with α-Adi3 (upper panel) or α-MBP (middle panel) antibodies. Lower panel, Coomassie stained blots indicating loading controls for plant extracts. (B) Cartoon representation of MBP-Adi3, the position of the MBP tag (green), the α-Adi3 peptide target (red) and the potential region in which Adi3 is cleaved (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

We reconstituted an *in vitro* ubiquitination system in which plant E1, E2s, and the AdBiL E3 were used. This system demonstrates that AdBiL has ubiquitin ligase activity *in vitro*. The *in vitro* ubiquitination of AdBiL is unexpected since RING E3 ligases are thought to act as scaffolding molecules bringing substrates and UBCs together, but do not incorporate activated Ub into themselves [3]. However, characterization of several RING E3s showed that auto-ubiquitination is a common, and perhaps auto-regulatory mechanism [25]. Both the significance of this auto-ubiquitination activity as well as the precise catalytic action of RING E3s is not known [26]. In this study, the autoubiqutination of AdBiL allowed for the identification of UBC8s from either tomato or *Arabidopsis* as the cognate E2s for AdBiL. Surprisingly, even closely related UBCs such as *At*UBC11 or *Sl*UBC10, failed to deliver Ub onto AdBiL. All of the UBCs analyzed are nearly identical at the amino acid level, except for a few polymorphic sites at residues 42, 47, and 133 (Supplemental Fig. 3C). Both UBC8s share an Ala at position 42 and a Ser at position 47, residues that are not conserved in either *Sl*UBC10 or *At*UBC11 (Supplemental Fig. 3B). This observation suggests that the differences in Ub transfer efficiency are caused by these two residues.

Ubiquitination assays with Adi3 by AdBiL showed that only a few Ub moieties are transferred to Adi3 by AdBiL *in vitro*. The number of Ub molecules is often an indicator of the fate of the protein. For instance, monoubiquitination can affect intracellular localization, whereas polyubiquitination can trigger proteasomal degradation [27]. Adi3 was rapidly degraded in the presence of plant extracts and this degradation was partially reversed with the addition of the 26S proteasome inhibitor MG132. This observation implies that Adi3 polyubiquitination is taking place. However, the direct involvement of AdBiL-mediated ubiquitination in the proteasomal degradation of Adi3 will require further testing.

The appearance of cleavage products when Adi3 is incubated with plant extracts suggests that proteases are involved in the degradation process (Fig. 4A lanes 2 and 6). During apoptosis, PKB is down-regulated at the protein level *via* ubiquitination and 26S proteasomal degradation. This degradation process depends on a type of pro-apoptotic cysteine-dependent proteases known as caspases [28,29]. Direct cleavage of PKB has been observed both *in vivo* and *in vitro* and inhibition of caspase activity *in vivo* completely abrogated the degradation of PKB [29]. Using caspase inhibitors plants have been shown to posses caspase-like activity [30]. This activity is now asserted to a group of caspases known as metacaspases which are involved in several regulatory processes including PCD [30]. The action of metacaspases could explain the partial MG132 protection observed in Adi3 cell-free degradation experiments (Fig. 4A lanes 1 and 12). Further testing should address whether metacaspase inhibition protects Adi3 from degradation. The protease calpain may also be involved in Adi3 degradation since MG132 also functions as a calpain inhibitor [31].

In addition to protein stability, ubiquitination is known to play a role in protein trafficking [2]. For instance, ubiquitinated PKB is recruited to the plasma membrane where it is activated by growth factor stimulation [32]. We previously showed that Adi3 PCD suppressing activity depends on its ability to transit from the plasma membrane into the nucleus in association with punctate membrane structures resembling endosomal vesicles [13]. Since it is known that protein monoubiquitination is a target for endosomal localization [33] it is possible that ubiquitination of Adi3 by AdBiL could be involved in regulating Adi3 localization.

Ubiquitination of PKB regulates its multiple functions by means of affecting protein stability, localization, and activation state. Adi3 ubiquitination as well as degradation resemble mechanisms used in PKB regulation and further supports Adi3 functioning similarly to PKB. Further characterization of Adi3 ubiquitination by AdBiL and its direct effect on protein stability and localization should help us understand the implication of this modification on Adi3 function in PCD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.043.

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