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Authors

Mark, Kevin G

Meza-Gutierrez, Fernando

Johnson, Jeffrey R

et al.

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Prb1 Protease Activity Is Required for Its Recognition by the F-Box Protein Saf1

Kevin G. Mark[†], Fernando Meza-Gutierrez[†], Jeffrey R. Johnson[‡], Billy W. Newton[‡], Nevan J. Krogan[‡], and David P. Toczyski^{†,*}

[†]Department of Biochemistry and Biophysics, University of California, San Francisco, California 94158, United States

[‡]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94158, United States

Abstract

The SCF ubiquitin ligase associates with substrates through its F-box protein adaptor. Substrates are typically recognized through a defined phosphodegron. Here, we characterize the interaction of the F-box protein Saf1 with Prb1, one of its vacuolar protease substrates. We show that Saf1 binds the mature protein but ubiquitinates only the zymogen precursor. The ubiquitinated lysine was found to be in a peptide eliminated from the mature protein. Mutations that eliminate the catalytic activity of Prb1, or the related substrate Prc1, block Saf1 targeting of the zymogen precursor. Our data suggest that Saf1 does not require a conventional degron as do other F-box proteins but instead recognizes the catalytic site itself.

Skp1-Cul1-F-box (SCF) ligases are multisubunit enzymes that mediate the destruction of regulatory proteins by attaching polyubiquitin chains to substrates, resulting in their recognition and elimination by the 26S proteasome.¹ SCF ligases share a common scaffold, an adaptor protein, and different F-box proteins that bind specific substrates.² The C-terminus of the F-box protein often contains either a leucine-rich repeat (LRR) or a WD40 repeat domain important for substrate interaction.³ In general, F-box proteins recognize substrates that contain posttranslational modifications, typically phosphorylation.⁴ Degradation motifs (degrons) are normally a single short stretch of amino acids⁵ or a series of such consensus sites.^{6,7} Degrons are usually N- or C-terminal extensions outside the catalytic domain and function when fused to ectopic substrates.^{8,9}

Saf1 is a poorly characterized F-box protein possessing RCC1 repeats, instead of an LRR or WD40 domain.¹⁰ Until recently, the only Saf1 substrate identified was the adenine-

* **Corresponding Author.** toczyski@cc.ucsf.edu. Telephone: (415) 502-1301. Fax: (415) 502-3179.

ASSOCIATED CONTENT

Supporting Information

Experimental details, Prb1 processing diagram, Saf1/Prb1 co-immunoprecipitations, and mass spectrometry spectrum. The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.biochem.5b00504](https://doi.org/10.1021/acs.biochem.5b00504).

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deaminating enzyme, Aah1, although its degron was not identified. Degradation of Aah1 occurs during entry of the cell into quiescence.¹¹

Recently, we identified a set of vacuolar serine proteases as Saf1 targets: Protease B (PRB1), Protease C (PRC1), and Ybr139w, a putative serine protease.¹² Vacuolar serine proteases play an essential role during starvation.¹³ *PRB1* encodes a 73 kDa protein precursor (preproPrb1) whose maturation involves at least four proteolytic cleavage steps (Figure S1 of the Supporting Information).¹⁴ After removal of the signal peptide (SS), 260 amino acids (P1) are removed from the N-terminus in an autocatalytic manner. The third cleavage, catalyzed by another vacuolar protease (Protease A), eliminates a small region of the C-terminus (P2). Finally, a 6 kDa peptide at the C-terminus (P3) is removed by autocatalysis to yield the 31 kDa mature protease (mPrb1). Prc1 processing is less well characterized.

The SCF^{Saf1} ligase associates with the polyubiquitinated form of preproPrb1.¹² To determine whether Saf1 selectively binds preproPrb1, we performed co-immunoprecipitations using cells expressing epitope-tagged Prb1 and Saf1. Note that C-terminally tagged Prb1 shows a delay in autocatalysis, thereby allowing precursor forms to accumulate.¹² We have previously found that the P1 region of Prb1 exhibits high levels of nonspecific binding to resin.¹² The F-box protein Grr1 was used as a control. Western blots of cell lysates were probed with an anti-Myc antibody that recognizes both preproPrb1 and proPrb1 and a polyclonal anti-Prb1 antibody that recognizes the first 14 amino acids of Prb1, present in all forms. Saf1 bound to the proPrb1 precursor (~75 kDa) at higher levels compared to that of the Grr1 control (Figure S2A of the Supporting Information). Surprisingly, Saf1 also bound significant amounts of mPrb1 (~31 kDa) (Figure 1A and Figure S2B of the Supporting Information).

Immunoprecipitations using a set of truncation mutants lacking various precursor fragments (mutants P1, P2–3, and P1 P2–3) showed that none of these *PRB1* mutants, including one containing only the sequences found in mature mPrb1 (P1 P2–3), were able to bind Saf1 (Figure S2C of the Supporting Information, data not shown). The inability of these forms to bind Saf1 is surprising because each of these truncated peptides contains mPrb1. These mutant forms of Prb1 are nonfunctional and were unable to complete zymogen processing.¹² These data suggest that proper folding of mPrb1 is critical for Saf1 binding.

It is possible that Saf1 recognizes preproPrb1, but rapid processing during or after immunoprecipitation resulted in the detection of only proPrb1 and mPrb1. To test this hypothesis, we performed a pulse-chase experiment by expressing *PRB1* under the inducible *GALI* promoter. Prb1 expression was induced with galactose for 15 min, and immunoprecipitations were then performed at various time points after induction. Western blot results showed an initial accumulation of a peptide corresponding to proPrb1 (40–42 kDa), followed by a conversion of this species into one that contains just P3 (37 kDa), with the eventual appearance of mPrb1 (Figure 1B and Figure S2D of the Supporting Information). Because detection of mPrb1 occurred only after the chase had been conducted for 30 min, this mature form of mPrb1 cannot result from autoproteolysis of a precursor form during purification. After the chase had been conducted for 10 min, several higher-molecular weight species can be seen in the Western blot, suggesting that Prb1 intermediates

were undergoing ubiquitination (see Figure 1B, IP lane). These data indicate processing is not occurring during or after immunoprecipitation and, surprisingly, that Saf1 binds preferentially to mPrb1 compared to its precursors.

We next investigated whether Saf1 binds mPrb1 in a cellular context or whether the interaction occurs upon cell lysis. To this end, we mixed equal volumes of cell lysates from a *Saf1-3xFlag; prb1* strain and a wild-type *PRB1* strain. As a positive control, we mixed cell lysates from a *Saf1-3xFlag; PRB1* strain with a *prb1* strain. In this case, the extract mixture contains the same amount of Flag-tagged Saf1 and mPrb1 originating from the same strain. Results from these experiments revealed that Saf1 binds mPrb1 in both cases, suggesting that the Saf1-Prb1 interaction can be reconstituted in the extract (Figure 1C).

One explanation for why Saf1 cannot bind truncated forms of Prb1 is that catalytic function is necessary to generate the correct conformation of the mature protease.¹² To examine this further, we mutated each catalytic residue (Asp325, His357, and Ser519) of Prb1 independently and examined polyubiquitination of these mutants using the Saf1 ligase trap.¹² Ligase trapping involves fusing an F-box protein to a ubiquitin-associated (UBA) domain, via a tandem Flag linker, to increase the affinity of the ligase for polyubiquitinated substrates. After anti-Flag immunoprecipitation, a Ni-NTA purification is performed under denaturing conditions, to enrich substrates conjugated to the six-His-tagged ubiquitin expressed in these cells. Results in Figure 2A show that Saf1 failed to precipitate the ubiquitinated forms of the D325N and the S519A mutants. Surprisingly, we found that the H357A mutant is capable of producing the proPrb1 form (Figure S3 of the Supporting Information), suggesting it has residual catalytic activity. Unlike the S519A and D325N mutants, the Saf1 ligase trap purifies ubiquitinated prb1-H357A. These data suggest that the ability to undergo catalytic processing is required for Prb1 recognition and ubiquitination by Saf1.

The fact that Prb1 recognition requires catalytic activity yet does not have to complete the first catalytic step suggests that proper ubiquitination might require that P1 be inside the substrate binding pocket in a cleavable form. To test this, we constructed a Prb1 mutant that blocks P1 cleavage by changing both the T280 and E281 residues flanking the cleavage site to prolines. The ubiquitinated T280P/E281P mutant failed to purify with the ligase trap (Figure 2A, data not shown). To determine whether this requirement for catalytic activity extends to other serine proteases, we examined the related protease, Prc1. Consistently, two independently derived strains with the analogous serine mutation in *PRC1* exhibited a reduced level of binding to the Saf1 ligase trap as a ubiquitinated species (Figure 2B).

To determine whether ubiquitination of catalytically inactive Prb1 can be rescued by the presence of wild-type Prb1 activity, we generated diploid Saf1 ligase trap strains containing wild-type *PRB1* and a Myc-tagged *prb1-S519A* mutant. Prb1 activity in the same cell did not allow ubiquitination of the catalytically inactive Prb1 mutant zymogen (Figure 2C), showing that mutant preproPrb1 cannot be rescued by Prb1 *in trans*.

To determine where ubiquitination occurs on Prb1, we affinity purified Prb1 from yeast cells using a Saf1 ligase trap. Following immunoprecipitation and affinity purification of cell

lysates using both the Flag and the His tags, ubiquitinated regions of Prb1 were mapped by the UbiScan procedure. UbiScan uses an antibody against branched diglycine (-KGG) motifs to enrich trypsinized extracts with ubiquitinated peptides prior to LC-MS/MS analysis. By this approach, a single ubiquitinated site was found at K325 of the P2/P3 region in two separate experiments (Figure S4 of the Supporting Information). Mutation of this lysine to arginine did not abrogate Prb1 ubiquitination (data not shown), suggesting that other lysines can be ubiquitinated.

In our initial ligase trap studies, we observed ubiquitinated full-length preproPrb1 because of its high abundance in cells carrying the Myc-tagged allele, which undergoes much slower processing. Subsequent analysis using an HA-tagged allele of Prb1 revealed a ladder of ubiquitinated forms of proPrb1 (Figure 3, right). These results are consistent with the finding that a significant amount of the ubiquitination occurs on the P2/P3 portion of this precursor molecule.

Saf1 may be the first example of an F-box protein that targets a specific class of enzymes based upon their catalytic sites. More commonly, F-box proteins recognize either diverse substrates or substrates involved in a general function, such as cell cycle regulation. However, in these cases, recognition typically occurs through a small, transferable degron. We propose that Saf1 recognizes a conserved protease structure that is shared among Prb1, Prc1, and Ybr139w. While we know of no other F-box proteins recognizing a class of molecules by their catalytic site, FBXO4 binds its target via a GTPase-like fold in the absence of posttranslational modification.¹⁵ Similarly, FBXL3 recognizes CRY1 and CRY2 (cryptochrome 1 and 2, respectively) via a conserved pocket on their surfaces.¹⁶

Given that the mature Prb1 protease is present only in the vacuolar lumen, it was an unexpected finding that Saf1 could bind mPrb1. We hypothesize that Saf1 recognizes the active protease domain of Prb1 in the context of the entire zymogen. Consistent with this hypothesis, we identified ubiquitination sites in P2/P3 of the Prb1 precursor (Figure 3 and Figure S4 of the Supporting Information). The fact that Saf1 can bind mPrb1 in cell extracts *in vitro* (Figure 1C) is consistent with a model in which mPrb1 is not the actual substrate targeted by Saf1 because it is missing the ubiquitination site and is in the wrong location, but still contains the recognition motif.

Where does SCF^{Saf1} target Prb1 in the cell? Because there are no ubiquitin enzymes or SCF components known to localize within the lumen of the endoplasmic reticulum (ER) or vacuole, we believe ubiquitination of Prb1 by Saf1 likely occurs in the cytosol. Presumably, SCF^{Saf1} binds Prb1 zymogen after it has retrotranslocated out of the ER.

Immunofluorescence experiments were not sufficiently sensitive to detect Saf1 (data not shown). It is possible that Sec61, the anterograde translocation pore, could be used as a channel for retrograde extraction of ubiquitinated Prb1 zymogen from the ER to the proteasome.^{17,18}

In conclusion, our studies of recognition of serine protease precursors by Saf1 suggest that this F-box protein binds its substrates either near the catalytic site or at a second site whose conformation is strongly affected by the catalytic site. Mutational analysis of this unusual

recognition is hindered by the fact that so many mutations block Prb1 activity. Thus, a thorough understanding of this binding event will need to await structural investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

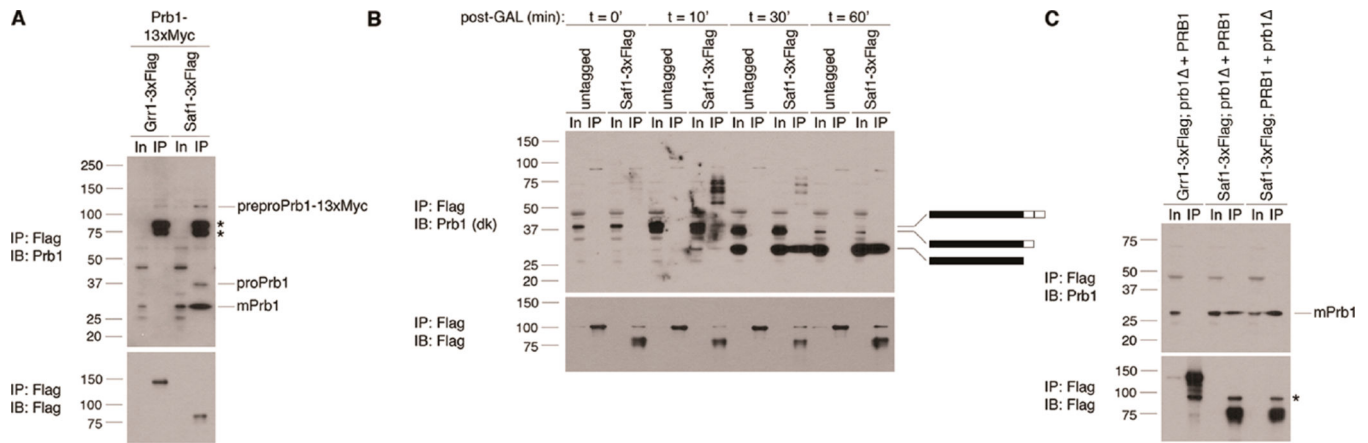
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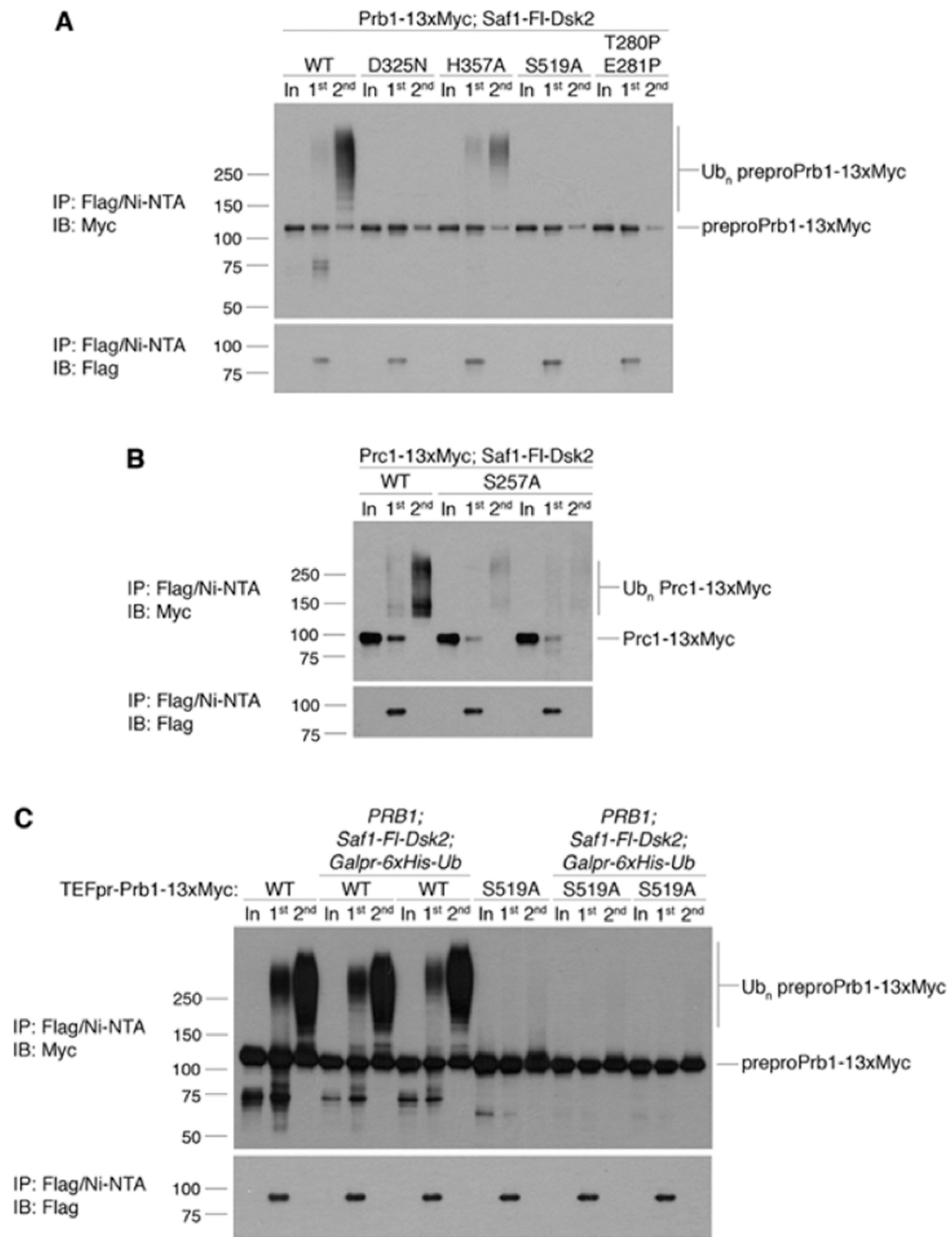
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**Figure 1.**

(A) Western blots of whole-cell extracts (In) and anti-Flag pull-downs (IP) from strains expressing Myc-tagged Prb1 and either Grr1-3xFlag or Saf1-3xFlag (asterisks denote nonspecific bands). (B) Strains expressing *PRB1* under the *GAL1* promoter were maintained in 2% raffinose and induced with 2% galactose for 15 min and collected at various time points, and cell lysates were prepared for anti-Flag pull-downs. (C) Different combinations of cell lysates were mixed prior to anti-Flag pull-down.

**Figure 2.**

(A) Two-step purifications from yeast strains expressing Prb1 mutants with compromised autocatalytic activities. Lanes are input (In), Flag immunoprecipitation (1st), and Ni-NTA purification (2nd). (B) Two-step purifications of wild-type and inactive (S257A) Prc1 protease, as in panel A. Two independent isolates of *prc1-S257A* are shown. (C) Prb1 ubiquitination in haploid yeast strains expressing wild-type (lanes 1–3) or mutant (lanes 10–12) Myc-tagged Prb1 vs diploid strains expressing wild-type (lanes 4–9) or mutant Myc-tagged (lanes 13–18) Prb1 with a second copy of untagged Prb1, Saf1 ligase trap construct,

and six-His-tagged ubiquitin. Two-step purifications as in panel A. Two isolates of each diploid are shown.

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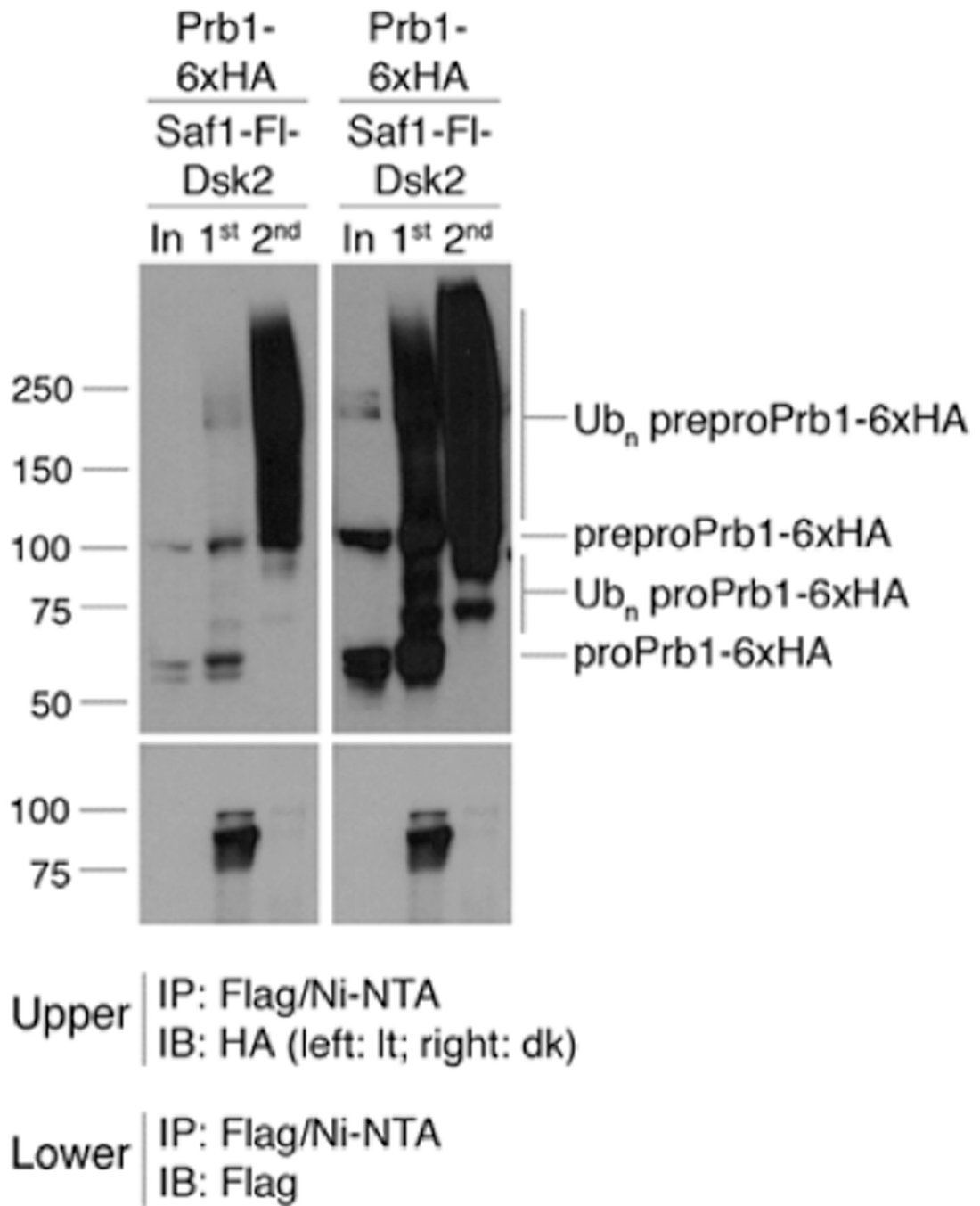


Figure 3. Purification as in Figure 2A, from a strain expressing HA-tagged Prb1. Light (left) and dark (right) exposures are shown. Laddering can be seen coming from both preproPrb1 and proPrb1.