[26] Two-Step Affinity Purification of Multiubiquitylated Proteins from *Saccharomyces cerevisiae*

By THIBAULT MAYOR and RAYMOND J. DESHAIES

Abstract

In budding yeast and higher eukaryotic genomes, there are, respectively, 50 and up to 400 or more distinct genes that encode for ubiquitin-ligases, and ~15–90 genes that encode for ubiquitin isopeptidases (TM and RJD, Semple *et al*., 2003). This puts ubiquitylation on par with phosphorylation as the most common reversible posttranslational modifications in eukaryotic cells. A key challenge that has met with limited success to date is to identify the proteins that are the substrates for this large collection of enzymes. To begin to address this daunting challenge, we sought to identify ubiquitylated proteins that are potential substrates of the 26S proteasome. Here, we describe a two-step affinity purification protocol that uses a budding yeast strain that expresses hexahistidine-tagged ubiquitin. In the first step, native cell lysate was chromatographed on a UBA domain-containing matrix that binds preferentially to K48-linked multiubiquitin chains. Free ubiquitin and presumably monoubiquitylated proteins did not bind this column, whereas proteins that are potential substrates of the proteasome were enriched. In the second step, UBA domain–binding proteins were subjected to immobilized metal ion affinity chromatography (IMAC) under denaturing conditions on magnetic nickel beads, resulting in >3000-fold enrichment of ubiquitin conjugates relative to crude cell extract.

Yeast Strain Design

A ubiquitin gene modified to encode an amino-terminal hexahistidine tag (H6-ubiquitin) was integrated into the *TRP1* locus of *Saccharomyces cerevisiae* W303 strain [*MATalpha, can1-100, his3-11,-15, trp1-1, ura3-1, ade2-1, leu2-3, -112*]. RDB1848 was created by placing H6-ubiquitin coding sequences between the GPD constitutive promoter and PGK terminator sequences of the modified yeast expression vector pG-1 (Schena *et al*., 1991) lacking the 2μ region (excised with EcoRI). Two primers (5′ GCGGATCCATGAGAGGTAGTCATCATCACCATCATCACGG TGGTATGCAGATTTTCGTCAAGACT 3′ and 5′ GAGCTCGAGAC-CACCTCTTAGCCTTAGCAC 3′) were used to amplify by PCR the yeast

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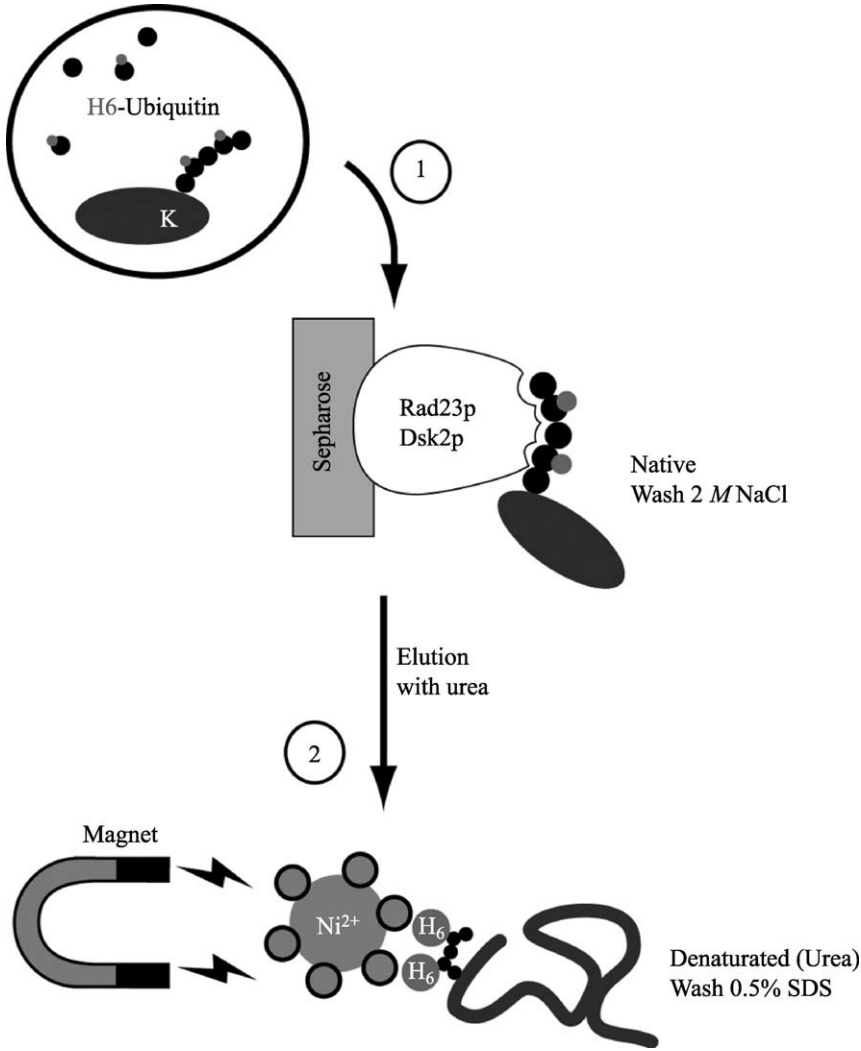

FIG. 1. Flow diagram for the two-step purification of multiubiquitin-conjugates. Yeast cells expressing a hexahistidine-tagged ubiquitin are lysed in a nondenaturing buffer, and proteins conjugated to multiubiquitin chains are purified using UBA domain–containing proteins (1). Proteins bound to the resin are washed with 2 *M* NaCl and eluted with urea. Denatured proteins are then mixed with magnetic nickel beads. Stringent washing conditions (up to 0.5% SDS) are used to wash away contaminants (2).

ubiquitin gene (first ubiquitin of the *UBI4* locus). The PCR fragment was digested with *Bam*HI and *Xho*I and ligated into the modified pG-1 (digested with *Bam*HI and *Sal*I). RDB1848 was then digested with *Eco*RV (which cleaves within *TRP1* sequences on the plasmid) and transformed into W303. Expression of H6-ubiquitin in RJD2997 was confirmed by Western blotting and corresponded to a 1:1 ratio with the unmodified ubiquitin (see Fig. 2B, first lane). The control strain RJD2998 was obtained by integrating the empty pG-1 vector into the *TRP1* locus.

Immobilization of Multiubiquitin Chain-Binding Proteins on Sepharose

We used recombinant Rad23p and Dsk2p proteins for the first step in our affinity purification scheme. Both proteins contain UBA domains that bind K48-linked ubiquitin chains with greater affinity than monoubiquitin or K63-linked chains (Raasi *et al.*, 2004; Wilkinson *et al.*, 2001). The glutathione S-transferase (GST) fusions of Dsk2 (pGEX-KG) and Rad23 (pGEX-6P1) were generous gifts from H. Kobayashi and H. Yokosawa, respectively. Fusion proteins were induced in BL21(DE3)/pLysS for 4 h at 30° with 1 m*M* IPTG and purified in sodium phosphate/Triton X-100 lysis buffer with Sepharose 4B resin following the manufacturer's instructions (Amersham Biosciences). Typically, 1 liter of induced bacterial culture yielded 10 to 15 mg of recombinant protein. Purified proteins were then dialyzed into 100 m*M* $NaHCO_3$, pH8.3, 0.5 *M* NaCl and coupled to CNBr-activated Sepharose 4B resin (Amersham Biosciences, ~10 mg of GST-Dsk2p and ~20 mg of GST-Rad23 were coupled each separately to 1.5 ml of resin). The recombinant proteins coupled to the resin could be stored for several months at 4° in a 50% slurry with 100 m*M* TrisHCl, pH8.0, 0.5 *M* NaCl, 0.02% NaN_3.

Two-Step Purification of Conjugates Bearing a Multiubiquitin Chain

1. The protocol described here is for 1 liter of yeast culture. Grow the cells at 25° in YPD (1% yeast extract, 2% peptone, 2% dextrose, optional 25 μg/ml ampicillin) until the OD_{600} reaches 1–1.5. Collect the cells in a 1-liter centrifuge bottle (Nalgene) using the H-6000A/HBB-6 rotor in a RC-3B Sorvall centrifuge run at 5000 rpm for 10 min. Wash the cells first with 200 ml ice-cold TBS (150 m*M* NaCl, 50 m*M* TrisHCl, pH 7.5), and then with 100 ml ice-cold TBS, 1 m*M* 1,10-phenanthroline (Sigma, P9375), 10 m*M* iodoacetamide (Sigma, I-1149, alternatively use 5 m*M* NEM, Sigma, E-3878). The latter two compounds are added to inhibit zinc- and cysteine-dependent ubiquitin isopeptidases. Iodoacetamide is preferred

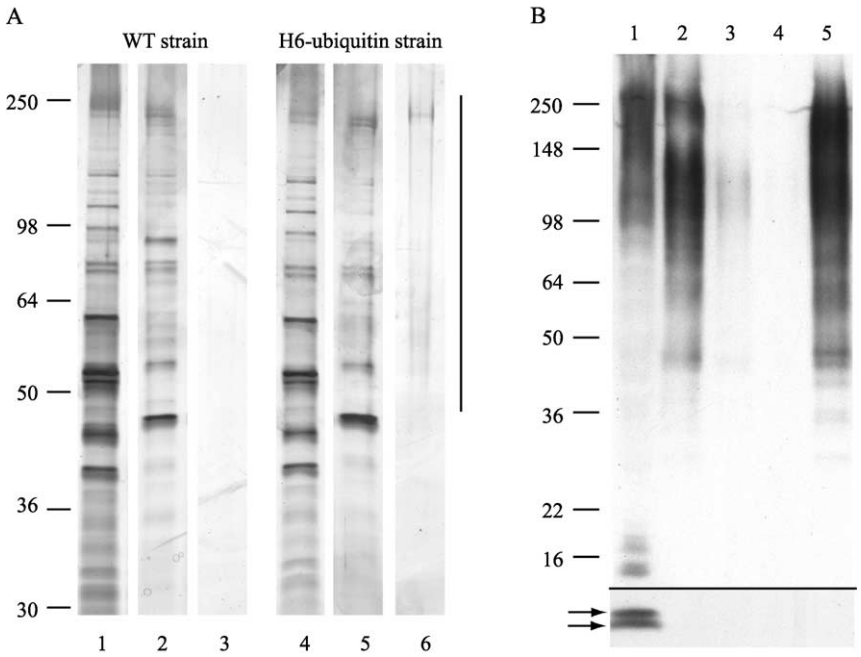

FIG. 2. (A) Silver staining of the two-step purification. Purifications were performed using the WT control strain (lanes 1–3) or the H6-ubiquitin expressing strain (lanes 4–6). Aliquots of total cell extract (lanes 1, 4), proteins eluted after the first step of the purification (lanes 2, 5), and proteins bound to the nickel magnetic beads (lanes 3, 6) were separated onto a 4–20% Tris-glycine PAGE. The fraction of each sample loaded was 4×10^{-7}(lanes 1, 4), 1/600 (lanes 2, 5), and 1/60 (lanes 3, 6). Lane 1 corresponds to 1 μg of loaded proteins. All the lanes were processed in the same way. (B) Immunoblotting of the two-step purification. Aliquot of total cell extract (lane 1), elution after the first step (lane 2), material that did not bind to the magnetic beads (lane 3), proteins washed by UB buffer containing 0.5% SDS (lane 4), and purified proteins after the second step of the purification (lane 5) were separated on a 4–20% Tris-glycine SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and blotted with an antiubiquitin antibody (mAb1510, ICN). The lower part of the gel corresponds to a lower exposure to allow visualization of distinct monoubiquitin and H6-ubiquitin bands. Lane 1 corresponds to a 10% input, lanes 4 and 5–5x more concentrated samples as compared with lanes 2 and 3. Note that only 25–30% of the purified ubiquitin conjugates were released from the magnetic beads in SDS-PAGE sample buffer.

over NEM if the sample is to be analyzed by mass spectometry. Freeze the cell pellet in liquid N_2 and store at –80°.

2. Thaw the cell pellet on ice for 5 min and add 14 ml of cold lysis buffer (50 m*M* sodium phosphate, pH 8.0, 300 m*M* NaCl, 0.5% Triton X-100, 0.5 m*M* AEBSF (MP Biochemicals, 193503), 5 μg/ml aprotinin,

5 μg/ml chymostatin, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 m*M* 1,10-phenanthroline, 10 m*M* iodoacetamide (or 5 m*M* NEM)). Lyse cells using a One Shot Cell Disrupter (Constant Systems) by applying 30,000 psi (foaming might occur). Collect the lysate as rapidly as possible and centrifuge at 4° in a Sorvall SS34 rotor for 20 min at 14,000 rpm. Alternately, cells can be lysed using the previously described mortar and pestle grinding method with liquid nitrogen (Verma *et al.*, 2000). Perform all the subsequent steps at 4°.

3. After clearing, collect the supernatant (typically 150–200 mg of proteins) in a 15-ml polypropylene conical tube and add 0.5 mg of each GST-Rad23 and GST-Dsk2 coupled to Sepharose (preequilibrated with lysis buffer). Mix gently for 90 min at 4° using a rotating wheel.

4. Centrifuge to pellet the resin. Use low velocity when centrifuging to prevent crushing of the resin (typically 1000 rpm for 5 min in a clinical centrifuge). Remove the buffer and wash the resin with 14 ml lysis buffer. Centrifuge, and then remove the buffer and add 14 ml of 50 m*M* sodium phosphate, pH 8.0, 2 *M* NaCl (avoid storing the 2 *M* NaCl solution directly on ice or alternately use a 1 *M* NaCl solution to prevent salt precipitation). Mix for 15 min using a rotating wheel. Centrifuge, remove the buffer, and perform one wash with 14 ml of 50 m*M* sodium phosphate, pH 8.0, 2 *M* NaCl, and two more washes with 14 ml of 50 m*M* sodium phosphate, 300 m*M* NaCl, 0.1% Triton X-100. If necessary, the washes can be performed in a gravity-flow column (e.g., Econo-Pac column, Bio-Rad).

5. The following steps are performed at room temperature. Elute the multiubiquitin conjugates by adding 2 resin-volumes of urea buffer (UB; 8 *M* urea, 100 m*M* NaH_2PO_4, 10 m*M* TrisHCl, pH 8.0). Mix well, centrifuge, and collect the supernatant. Repeat the procedure once and mix together the two elution fractions in an Eppendorf tube. Centrifuge for 2 min at 10,000 rpm in a Microfuge to pellet the residual resin and collect the eluate.

6. Add imidazole to the eluate to a final concentration of 20 m*M*. Add 25 μl of a magnetic nickel bead slurry (Promega, V8565) prewashed in the urea buffer (the actual bead volume should be around 7–8 μl). Mix gently for 60 min on a rotating wheel. Adapt the size of the tube (0.5, 1.5, or 2 ml) to the eluate volume to minimize loss of the nickel beads on the tube surface.

7. Wash the beads three times with 1 eluate-volume of UB. For the washes, place the tube in a magnetic rack for 10 sec, remove the supernatant, remove the tube from the rack, and mix the beads with a fresh aliquot of wash buffer.

8. If nonspecific background is a major concern, the wash protocol described here can be substituted for step 7. Remove the supernatant and

mix the beads with 1 eluate-volume of UB supplemented with 0.5% SDS. Incubate the beads for 15 min with gentle mixing and remove buffer. For mass spectrometry analysis, the SDS is extracted with Triton X-100. Wash the beads briefly with 1 eluate-volume of urea buffer with 0.5% Triton X-100, remove buffer, and add again 1 eluate-volume of urea buffer with 0.5% Triton X-100. Incubate for 15 min while mixing gently. Remove the buffer and repeat the same procedure with plain UB to remove the Triton. This detergent wash step may decrease the yield of the purification up to 20% but will greatly reduce the background.

9. After the washes, the proteins can be eluted with 500 m*M* imidazole or in sample buffer for PAGE analysis. For mass spectrometry analysis, we directly performed tryptic digests with the proteins bound to the beads (Mayor *et al*., 2005, see following).

Comments

We performed the two-step purification in parallel with a strain expressing H6-ubiquitin and a control strain that only expresses endogenous ubiquitin. Although a similar amount of proteins was recovered from both strains after the first step, no signal was detected in the control strain preparation on silver staining after the second step (Fig. 2A). By contrast, when the strain that expresses H6-ubiquitin was used, silver staining revealed a continuous spread of proteins ranging from 50–250 kDa. This is expected, because a collection of proteins of different molecular weights conjugated to multiubiquitin chains of variable lengths should produce a spread rather than a pattern composed of discrete bands.

In the first step of the purification, approximately 15% of the high molecular weight ubiquitin conjugates in the cell were recovered (Fig. 2B). It is important to note that ubiquitin conjugates were enriched, whereas the highly abundant monoubiquitin was not recovered. When starting with 150 mg of protein, we estimated that 20–50 μg of protein were eluted after the first step of purification, and some 3–5 μg remained bound to the nickel beads after the IMAC step (representing about 10% of ubiquitin conjugates in the cell). This represented a 3000- to 5000-fold enrichment for ubiquitin conjugates.

Our method provides an effective approach to purify conjugates that bear a multiubiquitin chain and can be adapted to study other related pathways. For example, purification can be biased in favor of monoubiquitylated proteins by using CUE or UIM domains instead of the UBA domain in the first step (Donaldson *et al*., 2003; Kang *et al*., 2003; Shih *et al*., 2002). A related method for the purification of ubiquitylated proteins has been described by Gygi and colleagues (Peng *et al*., 2003; see Chapter 25

in this volume). Our approach differs from that reported by Peng *et al.* in that their method uses a single-step purification of ubiquitylated proteins by IMAC, starting with a strain that contains H6-ubiquitin as the only source of ubiquitin. By contrast, our method uses a strain that expresses normal ubiquitin in addition to H6-ubiquitin and involves two consecutive affinity purification steps. The relative benefit of using a strain in which the tagged ubiquitin is the only source of ubiquitin, or is expressed in addition to normal ubiquitin, is not clear. On the one hand, our approach leads to an approximately two-fold overproduction of ubiquitin, which could potentially cause artifacts. On the other hand, in our experience tagged forms of ubiquitin are clearly less active than natural ubiquitin when the two are compared in reconstitution systems, and thus there may be less overall perturbation to the ubiquitin-proteasome system (UPS) if the tagged ubiquitin is diluted by the presence of natural ubiquitin (Rati Verma and RJD, personal communications). Indeed, we did not observe any increase of cadmium chloride (3 m*M*) sensitivity after integration of H6-ubiquitin (data not shown). By contrast, we believe that the inclusion of a purification step before IMAC is important, because even under denaturing conditions a significant fraction (0.5–1%) of total yeast extract proteins bind to Ni^{2+}–NTA resin. By using UBA domain affinity chromatography in tandem with IMAC, we were able to greatly enrich for multiubiquitin chain conjugates (>3000-fold).

When we analyzed the ubiquitylated proteins purified from 6 liters of culture by multidimensional chromatography-mass spectrometry (MudPIT or LC/LC-MS/MS), we routinely identified 150–200 proteins (Mayor *et al.*, 2005). Subsequent validation analyses enabled us to confirm the presence of a multiubiquitin chain on 7 proteins from a pool of 10 tested candidates expressed at their endogenous level. This strongly suggests that most of the purified proteins are *bona fide* substrates of the UPS. Using this purification method, we were also able to identify ubiquitylated proteins that specifically accumulated when part of the UPS system was impaired (i.e., deletion of the proteasome substrate receptor Rpn10). From this effort, we identified the cell cycle regulator Sic1 and the transcriptional activator Gcn4 as being candidate ligands for Rpn10. The fact that these two UPS targets are known to be present at low abundance underscores the power of our two-step affinity purification method (Mayor *et al.*, 2005).

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[27] Identification of SUMO–Protein Conjugates

By MEIK SACHER, BORIS PFANDER, and STEFAN JENTSCH

Abstract

Modification of proteins by covalent attachment of ubiquitin and the ubiquitin-like modifier SUMO are widespread regulatory events of all eukaryotic cells. SUMOylation has received much attention, because several identified targets play prominent roles, in particular, in cell signaling, gene expression, and DNA repair. Notably, only a very small fraction of a

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