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Binding of Ubiquitin Conjugates to Proteasomes as Visualized with Native Gels

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Summary

The proteasome is an ATP-dependent molecular machine that degrades proteins through the concerted activity of dozens of subunits. It is the yin to the ribosome's yang, and together these entities mold the protein landscape of the cell. Native gels are generally superior to conventional and affinity purifications for the analytical resolution proteasomal variants, and have thus become a staple of proteasome work. Here we describe the technique of using native gels to observe proteasomes in complex with ubiquitin conjugates. We discuss the consequences of ubiquitin conjugate length and concentration on the migration of these complexes, the use of this mobility shift to evaluate the relative affinity of mutant proteasomes for ubiquitin conjugates, and the effects of deubiquitinating enzymes and competing ubiquitin binding proteins on the interactions of ubiquitin conjugates with the proteasome.

Keywords

proteasome; ubiquitin; native gel; mobility shift; peptidase; protease; proteolysis

1. Introduction

Protein degradation by the proteasome influences nearly every aspect of cell biology. The proteasome is composed of the core particle, a barrel-shaped chamber with the active sites of the protease facing the interior, and regulatory particles, situated on either end of the core particle and preventing entry of proteins until substrates are properly engaged. Substrate engagement generally begins with the recognition of covalently attached ubiquitin molecules, a modification carried out by a trio of enzymes and often provoked by a shift in the physiological milieu of the cell. Once docked, the substrate can be more deeply engaged with the proteasome, and will ultimately undergo unfolding and translocation into the proteolytic chamber (1). In the course of degradation, ubiquitin is frequently regenerated by deubiquitinating enzymes, though in some cases these enzymes serve to shorten the ubiquitin conjugates on substrates and permit substrates to escape degradation (2,3). In the course of our work on ubiquitin recognition, we have investigated two types of ubiquitin receptors for the proteasome: extrinsic receptors, which deliver ubiquitinated substrates to the proteasome from the cytosol, and intrinsic receptors, which form the structure of the proteasome itself (4).

The work in our lab concerns many aspects of proteasome function, and we have found native gels to be broadly useful for proteasome analysis. The three most common forms of the proteasome, the core particle, the singly-capped holoenzyme, and doubly-capped holoenzyme, can all be resolved by native PAGE (5). These complexes can be subsequently assayed for peptidase activity in the gel. Using this technique, we have been able to discover subassemblies of the proteasomal regulatory particle (6), identify proteasome mutants that

are suppressed and enhanced for opening of the protein gate that seals the end of the core particle (7, 8), demonstrate the role of Blm10 in opening this gate (9), characterize proteasome assembly (10) and biogenesis (11, 12), and of course investigate the function of the proteasomal ubiquitin receptors (13–15).

This last depends on a native PAGE mobility shift induced by the association of proteasomes with ubiquitin conjugates. Notably, we have been able to detect the binding of the shuttling receptors by this method as well (13–15).

2. Materials

2.1 Protein expression

1. LB: 1% tryptone, 0.5% yeast extract, 1% NaCl
2. YPD: 1% yeast extract, 2% bactopectone, 2% dextrose
3. Rosetta 2 cells (EMD 71397-4)

2.2 Protease inhibitors

Prepare three separate cocktails of protease inhibitors, store them in single use aliquots at -20°C , and add each to lysis buffer just before preparing extract.

1. PIC 1, prepared in the aprotinin shipping solution (0.9% each NaCl and benzyl alcohol), (1000X stock)
 - 1 mg/ml leupeptin (Sigma L0649)
 - 2 mg/ml antipain (Sigma A6191)
 - 10 mg/ml benzamidine hydrochloride (Sigma B6506)
 - 5–10 TIU/ml aprotinin (Sigma A6279)
2. PIC 2, prepared in DMSO (1000X stock)
 - 1 mg/ml chymostatin (Sigma C7268)
 - 1 mg/ml pepstatin (Sigma P5318)
3. AEBSF, prepared in water (200X stock)
 - 200 mM AEBSF (Gold Biotechnology A5440)

2.3 Stock solutions

Stocks not described include buffers and salts which are stable indefinitely at room temperature.

1. ATP (Sigma A3377): 250 mM stock with 500 mM Tris base, 250 mM MgCl_2 , stored in single use aliquots at -80°C .
2. DTT (Sigma D9779): 1M stock, stored in single use aliquots at -80°C .
3. reduced glutathione (Sigma G6529): 500 mM stock, titrated to neutrality with NaOH and stored as single use aliquots at -20°C .
4. suc-LLVY-AMC (Bachem I1395): 10 mM in DMF, stored in small aliquots at -20°C .
5. acrylamide:bisacrylamide::37.5:1, 40% stock (BioRad 161-0148), stored at $+4^{\circ}\text{C}$.

6. imidazole (Sigma I2399): 2M stock, titrated to neutrality, and stored at room temperature.
7. 5X Native Gel Buffer: 450 mM Tris base with 450 mM boric acid; stable for one month at room temperature. Verify that the pH is 8.3.
8. APS (Sigma A3678): ammonium persulfate, 10% stock stored in single use aliquots at -20°C .
9. TEMED (Sigma T9281): N,N,N',N'-tetramethylethylenediamine, stored at $+4^{\circ}\text{C}$.

2.4 Chromatography resins and columns

1. fan-folded filter paper (VWR 28331-048)
2. Glutathione Sepharose (GE Healthcare 17-0756-01)
3. TALON resin (Clontech 635502)
4. IgG resin (MP Biomedicals 55961)
5. DEAE Fast Flow resin (GE Healthcare 17-0709-01)
6. Econocolumns with 1.5 cm diameter (BioRad 737-4151)
7. Mono Q HR5/10 (Pharmacia) or equivalent

2.5 Chromatography Buffers

Consult **Section 2.3** for relevant stocks.

1. Purification of GST fusion proteins (**Section 3.6**)
 - Buffer A1: 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT
 - Buffer A2: Buffer A1 with 10% (v/v) glycerol
 - Buffer A3: Buffer A2 with 25 mM glutathione
2. Purification of HIS-tagged Cdc34 (**Section 3.7**)
 - Buffer B1: 25 mM Tris-HCl (pH 8.0), 150 mM NaCl
 - Buffer B2: Buffer B1 with 10% (v/v) glycerol, 10 mM imidazole
 - Buffer B3: Buffer B1 with 10% (v/v) glycerol, 100 mM imidazole
3. Purification of Native Cdc34 (**Section 3.8**)
 - Buffer C1: 25 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM DTT
 - Buffer C2: 50 mM Tris-HCl (pH 7.5) and 0.5 mM DTT
 - Buffer C3: Buffer C2 with 100 mM NaCl
 - Buffer C4: Buffer C2 with 500 mM NaCl
 - Buffer C5: 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, and 10% (v/v) glycerol
4. Purification of HIS-Uba1 (**Section 3.9**)
 - Buffer D1: 50 mM HEPES-KOH (pH 7.3), 60 mM sodium acetate, 5 mM magnesium acetate, 1 mM DTT
 - Buffer D2: Buffer D1 with 100 mM KCl

Buffer D3: Buffer D1 with 500 mM KCl

Buffer D4: 50 mM sodium phosphate, 100 mM NaCl (pH 8.0)

Buffer D5: 50 mM sodium phosphate, 100 mM NaCl (pH 7.0)

Buffer D6: Buffer D5 containing 100 mM imidazole

Buffer D7: 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM DTT, and 10% (v/v) glycerol

5. Affinity purification of proteasome (**Section 3.10**)

Buffer E1: 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 6 mM MgCl₂, 1 mM ATP

Buffer E2: 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 6 mM MgCl₂, 1 mM ATP, 50 mM NaCl

Buffer E3: 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 6 mM MgCl₂, 1 mM ATP

2.6 Native PAGE

Consult **Section 2.3** for relevant stocks.

1. Hoefer Mighty Small SE 250 system for gel electrophoresis
2. Hoefer SE 275 or SE 215 multiple gel caster
3. gel loading tips (Denville P3080)
4. square petri dishes (10 cm, VWR 25378-047)
5. UV Transilluminator with 365 nm filter (UVP, see Note 1).
6. Imaging system
7. Gel Mix: 90 mM Tris base, 90 mM boric acid, 5 mM MgCl₂, 1 mM ATP, 0.5 mM EDTA, and 3.5% acrylamide.
8. Resolving Buffer: 90 mM Tris base, 90 mM boric acid, 5 mM MgCl₂, 1 mM ATP, 0.5 mM EDTA.
9. 5X Native Gel Loading Buffer: 250 mM Tris-HCl (pH 7.4), 50% (v/v) glycerol, 50 ng/ml xylene cyanol.
10. Reaction Buffer: 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, and 1 mM ATP.
11. Reaction Mix: 100 μ M suc-LLVY-AMC in Reaction Buffer.

2.7 Commercial proteins and protein management

1. bovine ubiquitin from red blood cells (Sigma U6253), prepared as a 500 mM stock in water (see Note 2).
2. AcTEV (Invitrogen 12575-015)
3. Coomassie Plus Protein Assay Kit (Pierce 23236)
4. Millipore concentrators, 10K cutoff (UFC801008)

¹AMC, the fluorescent cleavage product generated by proteasomal cleavage of suc-LLVY-AMC, has excitation and emission wavelengths of 365 nm and 440 nm, respectively. A UV filter transparent to 365 nm, rather than the more typical 302 nm, is essential for visualizing cleaved AMC.

²The concentration of a ubiquitin solution cannot be measured accurately by typical protein assays which use a BSA standard. On the other hand, the molar extinction coefficient is known ($\epsilon_{280} = 1254 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$), such that a 6.7 mg/ml solution will yield an A₂₈₀ of 1.

5. Millipore concentrators, 30K cutoff (UFC803008)
6. Snakeskin dialysis tubing, 10K cutoff (Pierce 68100)

3. Methods

3.1 Expression of fusion proteins in *E. coli*

1. Transform Rosetta 2 cells with plasmid encoding a HIS- or GST-tagged protein of interest, and grow overnight at 30°C in LB containing 50 µg/ml ampicillin. An intervening plating step may also be used.
2. Dilute overnight culture to a concentration of OD₆₀₀ of 0.15, and grow culture at 37°C until an OD₆₀₀ of 0.6–0.8 has been reached.
3. Shift cells to 30°C, add IPTG to a final concentration of 200 µM, and continue incubation for 4 hours to overnight.
4. Harvest cells by collecting in a chilled centrifuge rotor spun at 5,000 × *g* for 15 minutes. Pellets may be resuspended in small amounts of media and collected in smaller tubes as desired.
5. Proceed to extraction (**Section 3.4**), then purification (**Section 3.6 or 3.7**), or store pellets at –80°C.

3.2. Expression of native Cdc34 in *E. coli*

1. Grow AR58 cells bearing pNMCD34 (16), at 30°C in LB containing containing 50 µg/ml ampicillin to an OD₆₀₀ of 1 (see Note 3).
2. Shift cells to 42°C for 2 hours, then incubate for an additional 3 h at 39°C.
3. Harvest cells as described (**Section 3.1.4**), and proceed to extraction (**Section 3.4**), then purification (**Section 3.8**).

3.3 Expression of HIS-Uba1 in yeast

1. Inoculate strain JD77-1A (*uba1Δ::HIS3*) carrying pJD325 (pCUP1-HIS-Uba1) (17) into YPD containing 100 µM CuSO₄ at an OD₆₀₀ of 0.001.
2. Grow at 30°F for roughly 20 hours, until an OD₆₀₀ of 4 has been reached (see Note 4).
3. Harvest by spinning at 3,500 × *g* for 10 minutes in a chilled rotor.
4. Wash cells in ice cold water and proceed with extraction (**Section 3.4**), then purification (**Section 3.9**).

3.4 Preparation of extracts

1. Resuspend cell pellets at a ratio of 2–3 ml of buffer per gram of cell pellet, using lysis buffers as described for specific protein purifications (**Sections 3.6 – 3.10**). Be sure that frozen pellets are thawed completely.
2. Pour cell suspension into the chamber of a pressure cell and assemble.

³pNMCD34 expresses Cdc34 under the control of the λP_L promoter, and AR58 cells carry a temperature sensitive allele of the λ repressor.

⁴Many spectrophotometers have a linear range for yeast cultures that is narrower than that for bacteria. Our protocols assume that cultures are read in the linear range.

3. Set gauge pressure which corresponds to a chamber pressure of 15,000 psi for *E. coli* lysis, or 20,000 psi for *S. cerevisiae* lysis.
4. Use two passes to prepare extract, taking care that the gauge pressure does not drop below 90% of the setting during the course of lysis (see Note 5).
5. Clear extracts by spinning at $20,000 \times g$ for 25 minutes.
6. Pour supernatant through a funnel lined with fan-folded filter paper (**Section 2.4.1**) to clear lipids and particulate from extract.

3.5 Concentration and storage of proteins

Since the native gels that we use lack a stacking gel, the sensitivity of the assay relies on keeping the sample size small. We generally concentrate proteins before storage, which has the added advantage of increasing protein stability as long as aggregates do not form. In addition, complex formation and electrophoresis may be quite sensitive to buffer conditions. We store aliquots of matching buffer along with our purified proteins as assay controls.

1. Measure concentration of eluted protein with the BioRad assay (**Section 2.7.3**) as described by the manufacturer.
2. Using filtration devices from Millipore, concentrate proteins to a final concentration of 3 to 6 mg/ml. For proteasomes, use devices with a 30K cutoff (**Section 2.7.5**), and for all other proteins use devices with a 10K cutoff (**Section 2.7.4**). Concentration should be carried out at 4°C.
3. Store proteins in aliquots at -80°C, with the expectation that the proteins will be thawed no more than twice.

3.6 Purification of GST fusion proteins from *E. coli*

Lysis and all subsequent steps should be carried out 4°C.

- 1 Express proteins (**Section 3.1**), lyse cells in Buffer A1 supplemented with 1X protease inhibitors (**Section 2.2**), and clear extract as described (**Section 3.4**).
- 2 Incubate extract from 500 ml initial culture volume with 2 ml of glutathione resin for 1 hour with gentle tumbling (see Note 6).
- 4 Recover resin in an Econocolumn with diameter of 1.5 cm.
- 5 Wash resin by gravity with 20 bed volumes of Buffer A1, followed by 10 bed volumes of Buffer A2.
- 6 Elute with Buffer A3. We typically add one or two volumes of elution buffer, incubate for 5 minutes, collect eluent, and repeat this step until the protein concentration of the eluent falls below either 0.5 mg/ml or 25% of the most concentrated fraction.
- 7 Pool fractions containing protein, concentrate as required, and store as described (see **Section 3.5**).

3.7 Purification of HIS-tagged Cdc34 from *E. coli*

Lysis and all subsequent steps should be carried out 4°C.

⁵If the pressure drops significantly below 90% during lysis, an additional pass may be advisable. Even when pressed in the cold room, lysate temperatures increase with each pass, so lysis efficiency must be balanced against a temperature increase.

⁶GST fusion proteins vary broadly in the density at which they will saturate resin, and 2 ml of resin may not absorb all of the fusion protein present in extract prepared from 500 ml of culture. The optimal ratio depends on expression levels and the fusion protein itself.

- 1 Express proteins (**Section 3.1**), lyse cells in Buffer B1 with supplemented 1X protease inhibitors (**Section 2.2**), and clear extract as described (**Section 3.4**).
- 2 Incubate extract from 500 ml initial culture volume with 1 ml of TALON resin equilibrated with Buffer B1 for 1 hour at with gentle tumbling.
- 4 Recover resin in an Econocolumn with diameter of 1.5 cm.
- 5 Wash resin by gravity with 20 bed volumes of Buffer B1, then wash with 10 bed volumes of Buffer B2.
- 6 Elute with Buffer B3. For approach and criteria for elution, see **Section 3.6.6**.
- 7 Pool fractions containing protein, concentrate as required, and store as described (see **Section 3.5**).

3.8 Purification of unmodified Cdc34 from E. coli

Lysis and all subsequent steps should be carried out 4°C.

1. Express proteins (**Section 3.2**), lyse cells in Buffer C1 supplemented with 1X with protease inhibitors (**Section 2.2**), and clear extract as described (**Section 3.4**).
2. Prepare a DEAE column using 1 ml of resin per gram of cell pellet.
3. Equilibrate column with Buffer C2, then load extract onto the column.
4. Wash with 3 bed volumes of Buffer C2, followed by 3 bed volumes of Buffer C3.
5. Develop column using five bed volumes and a linear gradient beginning with Buffer C3 and ending with Buffer C4.
6. Pool fractions containing Cdc34 (detectable by a prominent 38kD band on SDS-PAGE stained with Coomassie; see (16) for enzymatic methods of detection), and dialyze into Buffer C2.
7. Subject to Mono Q HR 5/10, using the same column buffers and a similar gradient (18).
8. Dialyze into Buffer C5, concentrate as required, and store as described (**Section 3.5**).

3.9 Purification of HIS-Uba1 from yeast

Lysis and all subsequent steps should be carried out 4°C.

1. Resuspend washed pellet (**Section 3.3**) in Buffer D1 supplemented with 1X protease inhibitors (**Section 2.2**).
2. Lyse cells and clear extract as described (**Section 3.4**).
3. Prepare a DEAE column using 1 ml of resin per gram of cell pellet.
4. Equilibrate column with Buffer D1, and load extract onto the column.
5. Wash column with 10 bed volumes Buffer D2.
6. Elute proteins with 5 bed volumes of Buffer D3, collecting 10 fractions.
7. Pool fractions containing protein and dialyze against Buffer D4.
8. For each milligram of protein eluted from the DEAE column, equilibrate 0.2 ml of TALON resin with Buffer D4. Incubate resin with protein pool for 1 hour.
9. Recover resin in an Econocolumn with 1.5 cm diameter.

10. Wash resin 10 bed volumes with Buffer D4, followed by 5 bed volumes Buffer D5.
11. Elute protein with Buffer D6 using the approach described in **Section 3.6.6**. Adjust the eluent to contain 1 mM DTT and 10% (v/v) glycerol, adding the latter from an 80% (v/v) stock.
12. Concentrate as needed, dialyze against Buffer D7, and store as described (section **Section 3.5**).

3.10 Affinity proteasome purification

While mobility shift assays have been shown to work with conventionally purified proteasomes (13), affinity purification of proteasomes is by far more convenient (19), and more permissive for purification in the presence of destabilizing mutations (13). During the development of the affinity technique for purifying proteasomes, it was observed that three major salt-sensitive components copurify with the proteasome, including the deubiquitinating enzyme Ubp6 (19). As described below, we have observed that Ubp6 present during the conjugate binding assay can trim chains and affect the mobility shift, and proteasomes that are to be evaluated for ubiquitin conjugate binding should therefore be purified from strains in which the *UBP6* gene has been deleted (see Note 7).

Lysis and all subsequent steps should be carried out 4°C.

- 1 Grow yeast lacking Ubp6 and bearing a proteasome subunit tagged with Protein A at 30°C in YPD to an OD₆₀₀ of at least 4, and as high as 10 (see Note 4).
- 2 Harvest cells at $3,500 \times g$ in a chilled rotor, using small amounts of medium and an additional spin to pool cells into smaller centrifuge tubes as desired.
- 2 Resuspend cells in Buffer E1 at a ratio of 2–3 ml of buffer per gram of cell pellet, and prepare extract as described (**Section 3.4**). Protease inhibitors are not required, but in some instances may be desirable. The extract should have a pH of at least 7.0.
- 3 Incubate cleared extract with IgG resin at a ratio of 2 L initial culture volume per ml of resin for 70–90 minutes.
- 4 Recover resin in an Econocolumn with diameter of 1.5 cm.
- 5 Wash with 20 bed volumes of Buffer E2, followed by 10 bed volumes of Buffer E3.
- 6 Add 80 units AcTEV mixed with one bed volume elution buffer and incubate for one hour at 30°C, or for 16 hours at 4°C.
- 7 Elute cleaved protein with two or three bed volumes of elution buffer.
- 8 Concentrate as necessary, and store at –80°C.

3.11. Conjugate synthesis

Ubiquitination of proteins typically involves a trio of enzymes, namely the ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3), as well as a protein substrate. In the conjugation reaction, an isopeptide linkage is formed between the carboxylate at the C terminus of ubiquitin and the amine group on the lysine

⁷We have generated several Protein A tags for proteasome purification, appended to Pre1, Rpt1, and Rpn11, the last of which we use most frequently (19). Note that Rpn11 and Ubp6 are closely linked genes, and generating a strain carrying modifications at both loci through crossing must account for this.

side chain. This process is iterative, in that surface lysines on ubiquitin can be linked to the C termini of additional ubiquitin molecules, thus forming a chain. The ubiquitin conjugating enzyme Cdc34, unlike other E2s, has an interesting and useful property of building ubiquitin conjugates on itself, specifically using lysines near its C-terminus and well outside of the catalytic UBC domain. The canonical linkage for supporting protein degradation is through lysine 48 of ubiquitin, and autoubiquitination of Cdc34 proceeds through this linkage exclusively (16). A key feature for evaluating proteasome binding to ubiquitin conjugates is that Cdc34 does not appear to be degraded in the course of a binding reaction (20). Were it consumed to a detectable degree, a defect in binding could not be distinguished from a difference in the metabolism of ubiquitin conjugates.

1. Just before use, prepare a 25X stock of synthesis buffer: 500 mM Tris-HCl (pH 7.5), 250 mM MgCl₂, 2.5 mM DTT, 50 mM ATP (see **Section 2.3** for relevant stocks).
2. Prepare the ubiquitination reaction by combining, in the following order, and at the final concentrations indicated: water, 1X synthesis buffer, 50 μ M ubiquitin, 1–8 μ M Cdc34, and 1.5 μ M Uba1 (see Note 8).
3. Incubate for 15 – 20 hours at 30°C (see Note 9).
4. Evaluate the extent of ubiquitin conjugation by 4–12% SDS-PAGE developed in MES, or the formation of high molecular weight conjugates by 8% SDS-PAGE developed in Tris-glycine. In either case, follow modification by staining gel with Coomassie blue.
5. Store conjugates in aliquots directly at –80°C, or add glycerol to a final concentration of 10% (from and 80% [v/v] stock) to stabilize for longer storage (see Note 10).

3.12. Native PAGE

Native gels differ in their parameters from SDS-PAGE in that the latter resolves primarily on size, while native gels resolve proteins and protein complexes on the basis of three factors: size, native charge, and shape. In theory, it should be possible to evaluate any protein complex on native gels, assuming that the protein complex is intact in the buffer conditions for electrophoresis, and that the gel is run with the appropriate polarity and percentage acrylamide. Native gels are highly resolving for proteasome subcomplexes, achieving a resolution not accessible through other techniques. In addition, native gels have the advantage of using relatively small amounts of material; 10 μ g of proteasome, a small fraction of what can be purified from one liter of yeast culture, can be easily visualized (5).

1. Assemble minigel forms according to the manufacturer's instructions, using combs and spacers for 1.5 mm gels. Ensure that there are absolutely no leaks in the gel cassettes by testing the assembled chambers with water (see Note 11).
2. Prepare 12 ml of gel mix for each gel (**Section 2.6.7**).

⁸Tagged variants of Cdc34 may also be used to generate autoubiquitinated Cdc34. When comparing the degree of modification for a fixed concentration of Cdc34, GST-Cdc34 shows a greater degree of modification, and HIS-Cdc34 shows a more modest degree of modification.

⁹The degree of modification between 12 and 15 hours does not appear to be substantially different, though there is a significant difference in the extent of the mobility shift induced.

¹⁰Unlike ubiquitin, which is quite a robust protein, conjugated ubiquitin appears to form aggregates over time, even when stored at –80°C. Test conjugates shortly after synthesis, and maintain a stock of proteasomes to assay the behavior of conjugates over time.

¹¹We have used both Hoefer and BioRad apparati for pouring and running native gels. We find that the Hoefer gels give better band morphology on native gels, but it is also more difficult to prevent leaks. The BioRad apparatus leaks rarely, but shows more electrophoretic anomalies, particularly when two gels are run on the same electrode. A good method for circumventing the leaks attendant to the Hoefer cassettes is to use the multigel pouring chamber.

3. Add ammonium persulfate and TEMED (**Section 2.3**) each to a final concentration of 0.1%, and mix gently.
4. Slowly pipet the gel mixture along the inner edge of the spacer until the gel mixture reaches the top of the lower plate. Remove any bubbles that form and slowly insert comb into the gel, taking care not to trap air (see Note 12).
5. Incubate on the bench until polymerized, which will take about ten minutes.
6. Remove gel from the casting apparatus, mark the lanes with a waterproof marker, then remove the comb. Clamp plates to the electrodes used for running the gel, and fill the upper and lower chambers with cold Resolving Buffer (**Section 2.6.8**). Using a long narrow gel loading tip, straighten the well dividers as required, then place gel apparatus in the cold room.
7. Add 5X native gel loading buffer (**Section 2.6.9**) to samples at a final concentration of 1X and mix. Briefly microfuge to ensure that the entire sample is collected at the bottom of the tube.
8. Carefully load the gels with a P20 pipetman or similar (the action is smoother than that of a P200), using gel loading tips (**Section 2.6.3**) and releasing the sample near the bottom of the well.
9. Attach electrodes, and run from negative to positive at a constant voltage of 100 or 110 volts for 3 hours (see Note 13).
10. Fill a square petri dish (**Section 2.6.4**) with 20 ml of Reaction Buffer (**Section 2.6.10**). Remove the gel cassette from the electrodes, separate the plates, place the short end of the gel plate in the petri dish, and carefully dislodge the gel with reaction buffer administered from a transfer pipet.
11. Decant reaction buffer and replace with 15 ml of Reaction Mix (**Section 2.6.11**). Incubate for 13–15 min at 30°C. Carefully transfer to the UV filter with a spatula and photograph (see Note 14).

3.13. Conjugate binding

1. Ensure that all reaction components are concentrated enough to yield a final reaction volume of 16 μ l (see Note 15).
2. Incubate 4 pmol of proteasomes (usually from a stock concentration of 1 μ M or greater) with any proteasome ligands, subunits, or competitors (if used) in a volume of no more than 8 μ l. Incubate 5 minutes at room temperature. For all reactions not containing ligand, use an equal volume of matching buffer (see Note 16).

¹²Native gels are strongly susceptible to irregular polymerization dynamics, and pipeting the gel slowly along one of the spacers suppresses wild distortions that can obtain from pouring the gel rapidly down the center of the plate. Pouring gels in multigel casting chambers is trickier, given that gel sandwiches are bundled and the gel openings are less accessible to pipet tips. A syringe equipped with an 18.5 gauge needle can be used as a funnel for administering the gel. Position the needle about 2 cm into the gel sandwich, aligning the needle with the spacer and pointing the bevel pointed towards it, and hold in position with a ring stand and clamp.

¹³In two hours, the xylene cyanol dye front should reach the bottom of the gel. In three hours, the double-capped proteasome will have run roughly 20 mm into the gel.

¹⁴The heat of the UV lamp will promote the reaction, and the generated AMC will diffuse. The first images of the native gel are likely to be the sharpest.

¹⁵Reaction volumes of 16 μ l will result in a 20 μ l loading volume after the addition of 5X Native Gel Loading Buffer. Since these native gels lack stacks, protein samples are not compressed during the course of electrophoresis, and the larger the loading volume, the more diffuse the bands.

¹⁶Protein storage buffers may have an effect on proteasome mobility or activity. Adding a matching buffer to samples where ligands have not been added both eliminates volume differences that would otherwise arise and controls for the chemical composition of the buffer.

3. Mix proteasome samples from previous step with ubiquitin conjugates contained in a volume of 8 μ l or less. For reactions not containing ubiquitin conjugates, use 8 μ l of matching buffer, or conjugation mix without Cdc34.
4. Incubate for 15 minutes at 30°C.
5. Add one fifth volume of 5X Native Gel Loading Buffer (**Section 2.6.9**), mix, and spin in the microfuge as necessary. Resolve samples by native gel and develop as described (**Section 3.12**).

Our assay for probing the binding of ubiquitin conjugates to the proteasome depends on an altered mobility of the proteasome when associating with ubiquitin conjugates. The proteasome subcomplexes resolved with native gels are all expected to carry a negative charge under the conditions of the assay (Table 1), and we observe that complexes show a lower mobility than their constituent parts (Fig 1a). Like the proteasome itself, Cdc34 and ubiquitin are both predicted to carry a negative charge at the pH of the native gel (Table 1). As with proteasome subcomplexes, the combination of proteasome and ubiquitin conjugates induces a mutual mobility shift (Fig 1b). Since the conjugates alone do not give a signal in the peptidase assay, the mobility shift of the proteasome in the presence of ubiquitin conjugates is easily identified. We routinely observe that the core particle is not shifted by the addition of ubiquitin conjugates, and any CP present therefore serves as an internal control for the assay (see Note 17).

In the course of our work on the intrinsic ubiquitin receptors of the proteasome, we have tested a variety of ligands for their ability to induce a mobility shift of the proteasome on native gels. This was first attempted with GST-Rad23, and the strong mobility shift was used to localize the binding of Rad23 to the base of the proteasome (13). Notably, Rad23 cleaved from GST loses this ability (Marion Schmidt, personal communication), suggesting that the mobility shift supported by GST-Rad23 may depend on two proteasomes being bridged by a dimerized ligand. As for ubiquitinated Cdc34, we cannot rule out that the mobility shift does not depend on two proteasomes engaging a single ubiquitinated protein, either due to the self-association of Cdc34 (21) or the presence of more than one ubiquitin chain built on a single molecule of Cdc34 (16). We have also observed that unanchored ubiquitin conjugates cause a modest shift (13), suggesting that the presence of GST fused to a ligand is not essential for the mobility shift.

The degree of Cdc34 modification can be roughly controlled by varying its concentration in the conjugation reaction. Independently of its concentration, only about half of the Cdc34 appears to be conjugated with ubiquitin during the reaction (see Note 18, [16]). Assuming a nearly complete consumption of ubiquitin (Fig 2a), the number of ubiquitins on each molecule of Cdc34 can be estimated. As many as 100 molecules may be present on a single molecule of Cdc34 when present at a concentration of 1 μ M in the conjugation reaction, though perhaps not emanating from a single lysine (see Note 19). For Cdc34 conjugated at a concentration of 8 μ M, Cdc34 is modified with an average of 12.5 molecules of ubiquitin. This length variation in turn creates mobility shifts of different degrees (Fig 2b), with the shortest chains shown yielding the most modest shift, despite the concentration of conjugated ubiquitin being the same in every case. The binding capacity of the proteasome can also be titrated with different amounts of conjugates, rather than with different conjugate

¹⁷The peptide hydrolytic activity of the core particle is latent as compared with the activity in the context of the holoenzyme. The CP can be activated and hence better visualized on native gels by including 0.02% SDS when developing the native gels.

¹⁸Conversion of Cdc34 to modified forms was assessed by running unmodified Cdc34 at various concentrations on the same SDS-PAGE gel, and estimating the concentration based on the intensity of Coomassie blue staining.

¹⁹Uba1 may be modified with ubiquitin in the course of this reaction, and free chains may also form. A significant contribution of these factors would lower the estimate of Cdc34 modification, particularly for lower reaction concentrations of Cdc34.

lengths (Fig 2c). We anticipate the longest conjugates being particularly useful in demonstrating residual proteasome binding in variants lacking multiple ubiquitin receptors, and we have found shorter ubiquitin conjugates to be essential for demonstrating the formation of a ternary complex formed by the proteasome, Rad23, and ubiquitin conjugates (14), with the modest shift induced by shorter ubiquitin conjugates being enhanced by the addition of untagged Rad23.

The mobility shift assay was developed with the goal of distinguishing the strength of ubiquitin recognition between wild type and mutant proteasomes (see Note 20). It was by using this assay that we were able to show that the UIM of Rpn10, in the context of the proteasome, contributes to ubiquitin recognition (14). Moreover, we compared the mobility shift of mutant and wild type proteasomes with conjugates of several lengths, which yielded a consistent pattern of reduced mobility of the Rpn10-uim mutant proteasomes. Most recently, we have used the mobility shift assay to reveal Rpn13 as a proteasomal ubiquitin receptor (Fig 3, originally published in *Nature* 2008 (15)). Using moderately long conjugates, we observed that the mobility of the proteasome was modestly decreased by the absence of Rpn13, though in the context of rpn10-uim proteasomes, the mobility shift was all but eliminated (Fig 3a, (15)). The contribution of Rpn13 was further verified by adding recombinant Rpn13 back to proteasomes lacking Rpn13 and showing that recognition of conjugate was reconstituted (Fig 3c, (15)).

Substrates that are docked at the proteasome via ubiquitin conjugates can be degraded, but they can also be rescued from degradation by the deubiquitinating enzyme Ubp6 (2, 3). Using a catalytically inactive mutant of Ubp6, we have shown that Ubp6 does not contribute substantially to ubiquitin conjugate recognition by the proteasome (2), though stimulation of the gate by ubiquitin aldehyde does depend on Ubp6 (22). Since Cdc34 is not degraded by the proteasome, it is a strong candidate for Ubp6 trimming when docked at the proteasome. Indeed, we find that the presence of Ubp6 can lead to the shortening of ubiquitin conjugates, and a subsequent decrease in the observed mobility shift (Fig 4a, 4b). The safest course for avoiding artifactual results deriving from Ubp6 variations is to prepare proteasomes from strains deleted for the *UBP6* gene, an approach which has the added advantage of avoiding downstream perturbations caused by Ubp6. In addition to Ubp6, ubiquitin binding proteins may mask the affinity of the proteasome for ubiquitin conjugates by blocking binding. Using a variant of Rad23 lacking the proteasome-targeting UBL domain, we observe that this mutant receptor can titrate conjugates and block the mobility shift (Fig 4c; see also [23]). We anticipate this only to be a problem when using undefined mixtures of proteins. This type of competition with proteasomes for ubiquitin chain binding may be used deliberately, however, as a reciprocal assay for the strength of ubiquitin association.

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²⁰The validity of comparing mutants in this assay depends on a close similarity of the proteasome preparations, and this should be verified by SDS-PAGE. Proteasome complexes lacking the core particle, namely RP, lid, and base, will give no signal when evaluated with suc-LLVY-AMC cleavage. These complexes can be visualized by Coomassie blue staining or immunoblots of native gels.

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Figure 1. Mobility shift of the proteasome is induced by the addition of ubiquitin conjugates
(A) Schematic of native gel mobility shifts caused by assembly of proteasome subcomplexes. Black ovals denote assembled complexes, and grey ovals denote precursors. Throughout the figures, CP indicates core particle, RP indicates regulatory particle, and singly-capped and doubly-capped proteasomes are indicated by RP_1CP and RP_2CP , respectively. RP_c indicates the migration of the regulatory particle species that is competent to associate with the CP. This form is distinguished from RP_n , which migrates just below RP_2CP , and cannot associate with CP (10). Relative mobilities were derived from data presented here, as well as earlier data (10, 12). **(B)** Proteasomes (4 pmol) were incubated with ubiquitinated Cdc34 prepared using 1 μM Cdc34 (8 pmol, denoted “long”), or with ubiquitinated Cdc34 prepared using of 4 μM Cdc34 (32 pmol, denoted “short”), resolved by native gel electrophoresis, and evaluated by suc-LLVY-AMC hydrolysis. Top panel: suc-LLVY-AMC hydrolysis assay. Bottom panel: Coomassie blue staining of gel following fluorescent assay.

Figure 2. Variations in ubiquitin conjugate induced mobility shift as a consequence of chain length and concentration

(**A**) Ubiquitinated Cdc34 was synthesized with Cdc34 present at the concentrations indicated. Reaction mixtures (10 μ l of each) were resolved by 4–12% SDS-PAGE developed in MES, then stained with Coomassie blue. (**B**) *Titration of binding reactions with conjugates of different lengths.* Each synthesis mixture (8 μ l) was mixed with 4 pmol of proteasomes, incubated to allow for the formation of complexes, resolved by native gel electrophoresis, and evaluated by suc-LLVY-AMC hydrolysis. (**C**) *Titration of binding versus conjugate concentration.* Proteasomes (4 pmol) were incubated with various amounts of ubiquitinated Cdc34 prepared in reactions containing 2 μ M Cdc34, resolved by native gel electrophoresis, and evaluated by suc-LLVY-AMC hydrolysis.

Figure 3. Using mobility shift assay to probe for ubiquitin receptors

These data were originally presented in Nature 2008 (15), and are reproduced as sanctioned by Nature Publishing Group. Proteasomes were purified from cells lacking Ubp6, the three known shuttling receptors (Rad23, Dsk2, and Ddi1), and containing variations of Rpn10 and Rpn13 as indicated. **(A)** *Rpn13 and Rpn10 contribute to ubiquitin conjugate binding.* Proteasomes (4 pmol) were incubated with 16 pmol of ubiquitinated His-Cdc34 (synthesized with 2 μ M HIS-Cdc34), resolved by native gel electrophoresis, and evaluated by suc-LLVY-AMC hydrolysis. **(B)** Proteasomes from (A) (25 μ g) were resolved by SDS-PAGE and stained with Coomassie blue. An asterisk indicates a contaminating protein. **(C)** Reconstitution of ubiquitin binding in proteasomes lacking ubiquitin receptor Rpn13. Proteasomes (4 pmol) were incubated with Rpn13 or buffer, incubated to allow assembly, then incubated with ubiquitinated HIS-Cdc34 prepared as in (A). Samples were resolved by native gel electrophoresis, and evaluated by suc-LLVY-AMC hydrolysis.

Figure 4. Inhibition of the mobility shift assay

(A) Proteasomes (4 pmol) were incubated with 4 pmol of GST-Ubp6 or buffer, followed by incubation with 16 pmol of ubiquitinated Cdc34 (conjugated at 1 μ M) or buffer. Half of each sample was resolved on native PAGE and assayed by suc-LLVY-AMC hydrolysis. (B) The remaining half of each sample was resolved by 4–12% gradient SDS-PAGE developed in MES, and stained with Coomassie blue. (C) Proteasomes (4 pmol) were incubated with buffer or various amounts of GST- Δ UBL-Rad23 (last three lanes, containing 4, 8 and 16 pmol respectively), followed by incubation with 16 pmol of ubiquitinated Cdc34 (conjugated at 2 μ M) or buffer. Samples were resolved on native PAGE and assayed by suc-LLVY-AMC hydrolysis.

Table 1

Calculated size and charge of proteasome complexes and related proteins

protein or complex	size (kDa)	calculated charge at pH 8.3
RP ₂ CP	2577	−845
RP ₁ CP	1652	−528
RP	925	−317
lid	372	−120
base	523	−179
CP	727	−210
Cdc34	34	−46
ubiquitin	8.6	−0.8
Rad23	42.4	−42
GST	27.0	−3.6

For individual proteins, the sizes and charges were calculated using the protein sequences present in SGD (<http://www.yeastgenome.org/>) and Protein Calculator (<http://www.scripps.edu/~cdputnam/protcalc.html>). The charges generated by Protein Calculator are based on the Henderson-Hasselbalch equation and the following pK_A values: N-terminus (8.0), C-terminus (3.1), lysine (10.0), arginine (12.0), histidine (6.5), glutamic acid (4.4), aspartic acid (4.4), tyrosine (10.0), and cysteine (8.5). The core particle includes two each of fourteen subunits, including five beta subunits which are processed (24). The processed forms were used in the calculation. The base includes the six ATPases as well as Rpn1, Rpn2, and Rpn13. The lid includes Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, Rpn12, and Sem1. The RP includes all subunits of the base and lid, as well as Rpn10. The sizes and charges (evaluated for pH 8.3, the pH of the native gel) of the individual subunits were summed to give the size and charge of the various complexes.