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Role of C-terminal Extensions of Subunits β2 and β7 in Assembly and Activity of Eukaryotic Proteasomes*

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A close inspection of the crystal structure of the yeast 20 S proteasome revealed that a prominent connection between the two β-rings is mediated by the subunit β7/Pre4. Its C-terminal extension intercalates between the β1/Pre3 and β2/Pup1 subunits on the opposite ring. We show that the interactions promoted by the β7/Pre4 tail are important to facilitate the formation of 20 S particles from two half-proteasome precursor complexes and/or to stabilize mature 20 S proteasomes. The deletion of 19 residues from the β7/Pre4 C terminus leads to an accumulation of half-proteasome precursor complexes containing the maturation factor Ump1. The C-terminal extension of β7/Pre4, which forms several hydrogen bonds with β1/Pre3, is in addition required for the post-acidic activity mediated by the latter subunit. Deletion of the C-terminal tail of β7/Pre4 results in an inhibition of β1/Pre3 propeptide processing and abrogation of post-acidic activity. Our data obtained with yeast strains that expressed the mature form of Pre3 lacking its propeptide suggest that interactions between the Pre4 C terminus and Pre3 stabilize a conformation of its active site, which is essential for post-acidic activity. Deletion of the C-terminal extension of β2/Pup1, which wraps around β3/Pup3 within the same β-ring, is lethal, indicating that this extension serves an essential function in proteasome assembly or stability.

Ubiquitin-mediated proteolysis is the main pathway for ATP-dependent non-lysosomal degradation of intracellular proteins in eukaryotes (1, 2). Polyubiquitylated substrates are recognized and degraded to small peptides by a multisubunit protease termed 26 S proteasome. This large proteolytic complex is composed of a 20 S barrel-shaped complex (the 20 S proteasome) that encloses the proteolytic sites, to which at both ends 19 S regulatory complexes are attached. The latter harbor isopeptidase and ATPase activities as well as binding sites for polyubiquitin chains (3–5).

20 S proteasomes are present in all eukaryotes, as well as in some archaea and eubacteria. In 1995, the structure of the 20 S proteasome of the archaea *Thermoplasma acidophilum* solved by x-ray crystallography was reported. This proteasome is composed of two distinct types of subunits, α and β, that are organized in four heptameric stacked rings with the arrangement $\alpha_7\beta_7\beta_7\alpha_7$. The β-rings form a central cavity that contains 14 active sites located on the inner surface (5). Two years later the crystal structure of the eukaryotic *Saccharomyces cerevisiae* 20 S proteasome was determined revealing a similar organization. However, the two identical halves contain seven different α-type subunits and seven distinct β-type subunits occupying unique locations within the structure. Only three of the seven different β-subunits are active (6). Based on mutational and structural studies it was found that subunits β1/Pre3, β2/Pup1, and β5/Pre2 harbor the peptidase sites, which mediate activities classified as post-acidic, tryptic, and chymotryptic, respectively (6–9). In mammalian proteasomes, the constitutive active subunits β1, β2, and β5, upon immune stress, can be replaced by the interferon-γ-induced subunits β1i, β2i, and β5i, respectively, resulting in the formation of so-called "immunoproteasomes" (10). More recently, the crystal structure of a constitutive mammalian 20 S proteasome was determined showing that the overall structure of eukaryotic proteasomes is highly conserved (11).

In all proteasomes studied, α-type and β-type subunits share a common "β-sandwich" fold, in which two antiparallel β-sheets are sandwiched in between two layers of α-helices (3). Major structural differences exhibited by the individual subunits, such as insertions or C- and N-terminal extensions, are important features that determine the specific subunit interactions within the α- or β-rings (*cis* contacts) and between the rings (α-*trans*-β and β-*trans*-β contacts). These interactions target each of the 14 subunits to unique locations within the structure of the eukaryotic 20 S proteasome (6, 11).

In *Thermoplasma*, the β-subunits are synthesized as precursors that require posttranslational processing for activation. Similarly, all active eukaryotic β-type subunits are synthesized as inactive precursors with N-terminal propeptides. These propeptides are cleaved off by intrasubunit autolysis yielding the mature forms with N-terminal threonine residues that act as nucleophiles attacking peptide bonds of substrates (7, 12–14). This property places proteasomes into the family of "Ntn hydrolases" that is characterized by an N-terminal nucleophile (15, 16).

Another structural aspect of several β-subunits is their C-terminal extensions that are involved in interactions within or across the rings. Subunit β2 has the longest C-terminal extension (37 residues) with which it embraces β3 within the same ring. The C-terminal extension of β7 resembles a clamp that projects from one half of the proteasome to reach out to the opposing half entering into its β-ring between β1 and β2. In the present study, we have investigated the role of the C-terminal extensions of β2 and β7. We present evidence showing that, in yeast, the β7/Pre4 C-terminal domain has a role in mediating

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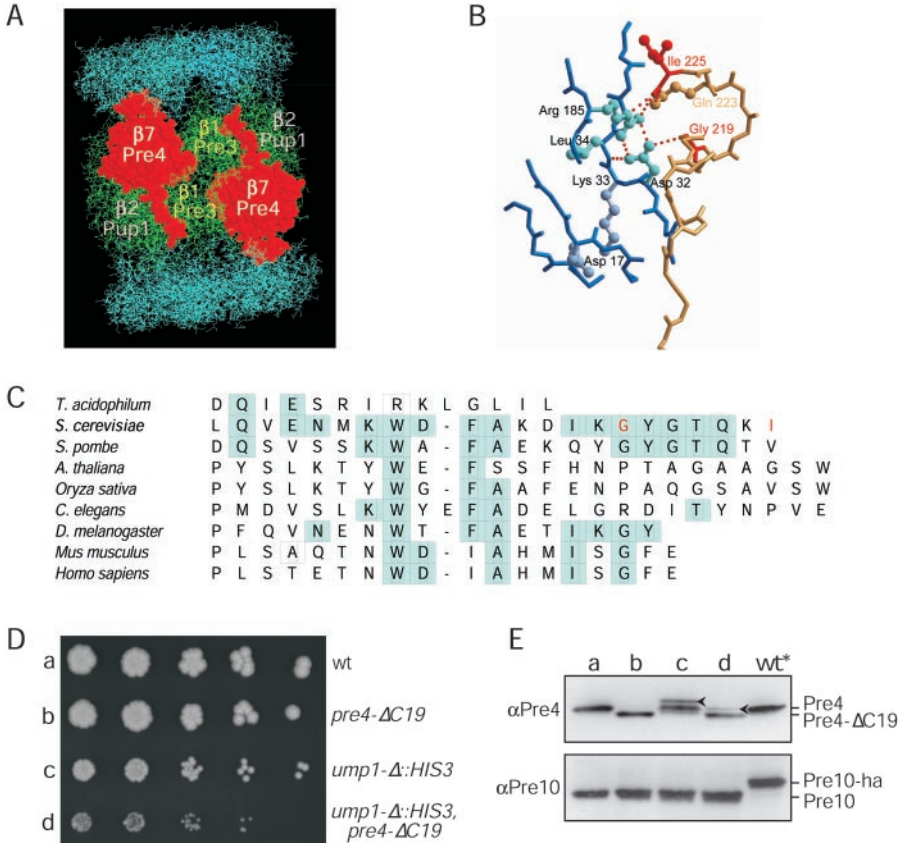

FIG. 1. **Structural aspects of the β7/Pre4 C terminus tail.** *A*, structure of *S. cerevisiae* 20 S proteasome emphasizing the β7/Pre4 β-*trans*-β interactions with β1/Pre3 and β2/Pup1. This figure was prepared with RasMol 2.6. β7/Pre4 is shown in the spacefill representation; all other subunits are displayed as wireframe. *B*, detail of the contact sites between the β7/Pre4 C-terminal extension (*orange* and *red*) and β1/Pre3 (various shades of *blue*). The prediction of hydrogen bonds (shown as *dotted lines*) and the graphic representation were done using SETOR (25). Side chains of relevant residues are highlighted as balls and sticks. *C*, amino acid sequence alignments of the C-terminal regions of β7 subunits of the indicated species. Residues identical between β7/Pre4 from *S. cerevisiae* and other species are *boxed*. *D*, synthetic growth inhibition by *ump1*-Δ and *pre4*-Δ*C19*. Spore clones (*a–d*) derived from JD281 (Table I) with the indicated genotypes were spotted in serial dilutions onto YPD medium and incubated at 30 °C for 2 days. *wt*, wild type. *E*, analysis of the level of two proteasomal subunits, β7/Pre4 (*top*) and α7/Pre10 (*bottom*), in the same strains (*a–d*) as in *D* with anti-Pre4 and anti-Pre10 antibodies, respectively. Strain JD183 expressing a slower migrating Pre10-ha (*wt**) was included as a control to demonstrate specificity of the anti-Pre10 antibodies. 30 μg of crude extract proteins were loaded/lane. The propeptide-containing precursor forms of Pre4 and Pre4-ΔC19 detected in the *ump1*-Δ background are indicated by *arrowheads*. For the wild-type *UMP1* strains, these forms were detectable only upon long exposure of the immunoblot (data not shown).

efficient generation of 20 S proteasome from its precursors. In addition, we show that specific interactions between this domain and β1/Pre3 are essential for post-acidic peptidase activity. The C-terminal extension of β2/Pup1 is essential for yeast cell viability indicating that functional proteasomes cannot form in its absence.

EXPERIMENTAL PROCEDURES

Yeast Media—Yeast-rich (YPD) and synthetic (SD) minimal media with 2% dextrose were prepared as described previously (17).

Construction of Yeast Strains and Plasmids—The yeast strains used in this study are listed in Table I. Construction of chromosomal open reading frames that expressed C-terminally truncated versions of β7/Pre4 was performed as follows. Different 3′-portions of *PRE4* were generated by PCR using primers containing flanking EcoRI and XbaI sites. The 3′-primers were designed such that the codons for Ile^{225}, Gly^{219}, or Glu^{207} (residue numbers refer to the processed subunits according to Ref. 6) were replaced by the stop codon TAA to produce β7/Pre4 mutant proteins lacking one (*pre4*-Δ*C1*), seven (*pre4*-Δ*C7*), or 19 (*pre4*-Δ*C19*) amino acid residues at the C terminus, respectively. The PCR fragments were cloned into the integrative plasmid YIplac204 (*TRP1*-marked) (18). All plasmids were verified by DNA sequencing. The resulting plasmids were linearized with BglII within the coding sequence for targeted integration into the *S. cerevisiae* genome. The generation of a chromosomal allele expressing a truncated version of β2/Pup1 lacking 30 residues at the C terminus (*pup1*-Δ*C30*) followed a similar strategy except that EcoNI was used for linearization. To follow the truncated Pup1, another genomic allele was generated that expressed a fusion with two *ha* epitopes (Pup1-ΔC30-ha) by an analogous strategy. The integrative plasmids that were used to generate in-frame fusions of full-length *PUP1* and *PRE3* with a sequence encoding two *ha* epitopes were described earlier (19). The same strategy was used to produce a genomically tagged strain expressing α7/Pre10-ha.

To express a β1/Pre3 protein that was identical to the mature processed form, the propeptide-encoding region of *PRE3* gene was replaced in the genome, by a two-step gene transplacement protocol, with a sequence encoding ubiquitin (Ub)[1] (details available upon request). The Ub-Pre3 protein synthesized by the resulting strain is processed co-translationally or nearly so by Ub-processing proteases (20). To exclude posttranslational acetylation of the N-terminal Thr residue of Pre3 generated from a Ub-Pre3 fusion, yeast strains with chromosomal deletions of *NAT1 (nat1*-Δ::*KanMX4*) were generated (21). *NAT1* encodes a subunit of N-terminal acetylase responsible for this modification (20).

Fractionation of Whole-cell Extracts by Gel Filtration, Electrophoresis, and Immunoblotting—*S. cerevisiae* cells were grown at 30 °C in SD medium to A_{600} of 1.2 ± 0.2 and treated as described by Ramos *et al.* (19). Protein extraction and fractionation on a Superose 6 column coupled to an ÄKTA FPLC (Amersham Biosciences) as well as SDS-PAGE and immunoblots were performed as described previously (19). Native PAGE was carried out according to published protocols (22). Polyclonal rabbit antibodies raised against β7/Pre4 were a kind gift from the laboratory of Dieter Wolf. Polyclonal anti-α7/Pre10 antibodies were raised against Pre10-His_6 expressed in *Escherichia coli*.

Proteasomal Peptidase Activity Assays—Determination of proteasomal activities in crude extracts was performed as follows. Yeast cells

[1] The abbreviations used are: Ub, ubiquitin; CP, core particle; R, regulatory particle.

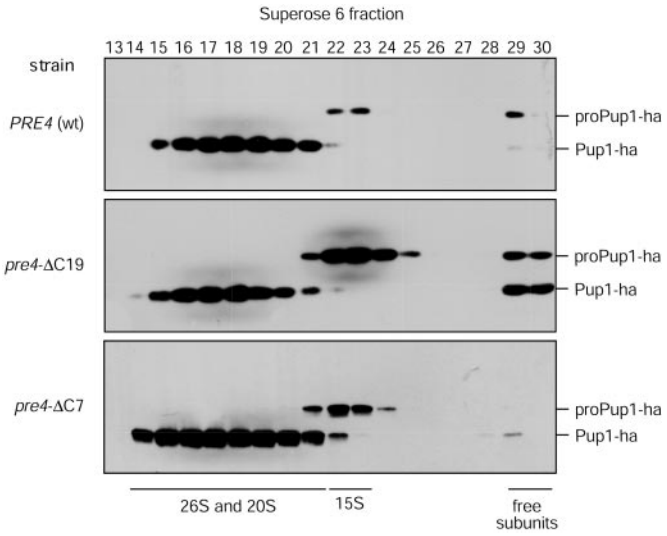

FIG. 2. **C-terminal truncations of β7/Pre4 inhibit the assembly of 20 S proteasomes.** Proteasomal complexes were analyzed by gel filtration of crude extracts and detection of β2/Pup1-ha by immunoblotting. The congenic yeast strains were JD139 (*PRE4*), JD234 (*pre4-ΔC19*), and JD320 (*pre4-ΔC7*) (Table I). *wt*, wild type.

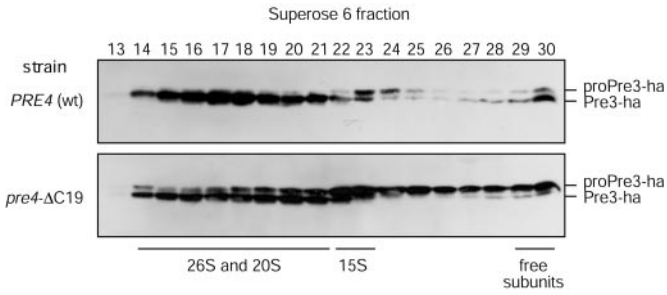

FIG. 3. **C-terminal truncation of β7/Pre4 inhibits β1/Pre3 processing.** Proteasomal complexes were analyzed by gel filtration of crude extracts and detection of β1/Pre3-ha by immunoblotting. The congenic yeast strains were JD301-3C (*PRE4*) and JD301-5A (*pre4-ΔC19*) (Table I). *wt*, wild type.

from exponentially growing cultures (A_{600} = 0.8–1.2) were harvested at 3000 × *g*, washed with cold water, frozen in liquid nitrogen, and stored at −80 °C. The cells were lysed with acid-washed glass beads (∅ 0.4–0.5 mm, Sigma) in extraction buffer (50 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 15% glycerol) by vortexing. Cell debris was removed by centrifugation at 15,800 × *g* at 4 °C. The protein content in the supernatant was determined using the Bio-Rad Bradford protein assay. For assaying chymotrypsin-like activity, reactions were set up with 5 μg of extract protein and 5 μg of succinyl-Leu-Leu-Val-Tyr 7-amido-4-methylcoumarin (Bachem) as substrate. The trypsin-like activity and the post-acidic activity were measured using 20 μg of protein and 5 μg of *t*-butoxycarbonyl-Leu-Arg-Arg 7-amido-4-methylcoumarin (Affinity) and Ac-Gly-Pro-Leu-Asp 7-amido-4-methylcoumarin (Bachem) as substrates, respectively. The assay conditions were described previously (19).

RESULTS

Structure of the β7/Pre4 C-terminal Tail and Its Interactions with β1/Pre3—The overall architecture of the 20 S proteasome and the general structures of the α- and β-subunits are conserved between *T. acidophilum* (5), *S. cerevisiae* (6), and mammals (11). One striking structural difference between the bacterial and the eukaryotic proteasome is confined to the β7 subunit. Inspection of the crystal structure of the yeast proteasome reveals a long C-terminal domain of β7/Pre4. This tail is composed of 19 amino acid residues and extends from one half of the proteasome to the other where it ends between the subunits β1/Pre3 and β2/Pup1, the former of which carries the active site for post-acidic peptidase activity (Fig. 1*A*). Both the carboxyl group of the last residue Ile^{225} and the side chain of Gln^{223} of β7/Pre4 form hydrogen bonds with the side chain of Arg^{185} of β1/Pre3 (residue numbers refer to the processed subunits according to Ref. 6). Arg^{185} within Pre3 together with Gly^{219} of β7/Pre4 form hydrogen bonds stabilizing Asp^{32} of Pre3, which itself forms a hydrogen bond with Leu^{34}. The latter two residues are direct neighbors of Lys^{33}, which is part of the post-acidic catalytic site, suggesting that this network of hydrogen bonds may be important for post-acidic activity (Fig. 1*B*). A comparison of the C-terminal sequence of β7 subunits from various species (Fig. 1*C*) revealed that its extension is only moderately conserved among eukaryotes.

The C-terminal Extension of β7/Pre4 Facilitates the Formation of 20 S Particles from Two Precursor Complexes—During our studies on the *ump4-1/pre4-3* mutant, in which a residue of the β7/Pre4 subunit was exchanged that is close to the site where its C-terminal extension emanates from one β-ring to reach out to the other, we found that proteasome precursor complexes accumulate.[2] Prompted by this finding we asked whether a defined deletion of the C-terminal tail of β7/Pre4 would be inhibitory to the formation of 20 S proteasomes from two 15 S half-proteasome precursor complexes. These precursor complexes are characterized by the presence of the propeptide-bearing precursor forms of the active β-type subunits, β1/Pre3, β2/Pup1, and β5/Pre2, and the proteasome maturation factor Ump1 (19). We generated a strain in which the C-terminal 19 amino acid residues of β7/Pre4 were deleted (*pre4-ΔC19*). In the same strain, a double *ha* epitope tag was linked to the C terminus of β2/Pup1. To examine whether the C-terminal truncation of Pre4 caused changes in the intracellular ratios of proteasomal precursor complexes and mature proteasomes, we analyzed cell extracts of the *pre4-ΔC19* mutant by gel filtration on a Superose 6 column. As for the wild type, the half-proteasome precursor complex of the *pre4-ΔC19* eluted with a peak in fractions 22 and 23 (Fig. 2). The ratio of this precursor complex to mature proteasomes, however, is significantly higher in the mutant (Fig. 2, *middle panel*) resulting also in a spreading of the immunoblot signal from fractions 21 to 25. As described previously (19), the mature form of β2/Pup1 is present in earlier fractions containing proteasomal peptidase activity due to the presence of assembled and active 20 S and 26 S proteasomes. Noteworthy is also the observation that the mature form of β2/Pup1 is in addition detected as a free subunit in fractions 29 and 30 of *pre4-ΔC19* mutant extracts (Fig. 2). In contrast, no free mature β2/Pup1 was detected in these fractions of wild-type extracts. These findings suggest that the free processed subunits observed in the *pre4-ΔC19* are derived from matured 20 S proteasomes that fell apart, either *in vivo* or *in vitro*.

Similar experiments were performed with wild-type and *pre4-ΔC19* strains expressing β1/Pre3 tagged at the C terminus with two *ha* epitopes. Proteins extracted from these strains were fractionated by gel filtration on Superose 6. Again, we observed a strikingly increased amount of β1/Pre3 precursor form in fractions 22 and 23 in the *pre4-ΔC19* mutant in comparison with the wild type (Fig. 3). In contrast to the β2/Pup1 precursor, however, the β1/Pre3 precursor form is in addition present in a wide range of fractions ranging from those containing the free subunit to those with 20 S and 26 S proteasomes. Particularly interesting was the simultaneous presence of β1/Pre3 mature and precursor forms in the fractions containing 20 S and 26 S proteasome suggesting a defect in β1/Pre3 maturation.

[2] M. K. London, B. I. Keck, P. C. Ramos, and R. J. Dohmen, manuscript in preparation.

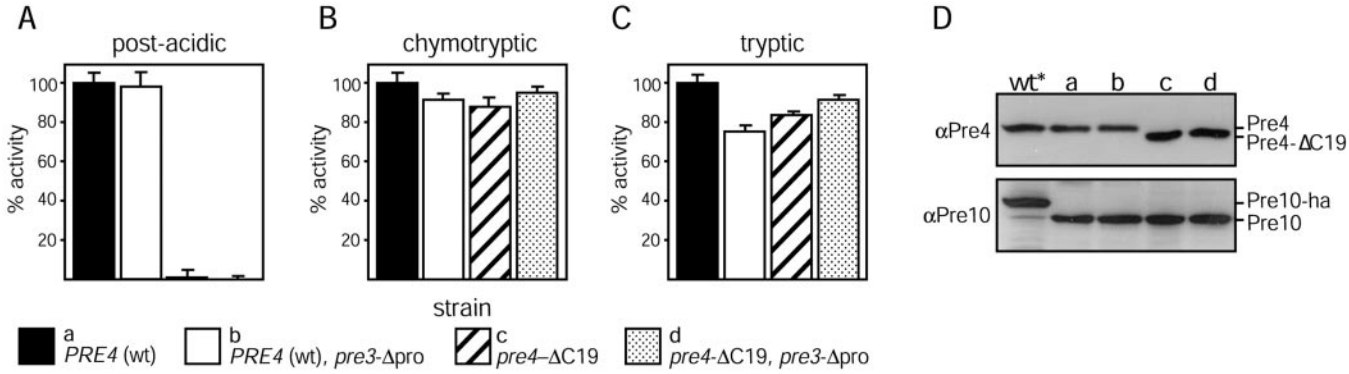

FIG. 4. **The β7/Pre4 C-terminal tail is essential for the post-acidic peptidase activity independent of β1/Pre3 propeptide processing.** Proteasomal peptidase activities (*A–C*) in the crude extracts of strains with the indicated genotypes were determined as described under "Experimental Procedures." The values were normalized to the levels in the wild-type (*wt*) strain, which were set to 100%. The strains used were JD284-6D (*a*), JD300-12C (*b*), JD300-3D (*c*), and JD300-5C (*d*) (Table I). All these strains carried a chromosomal deletion of *NAT1 (nat1*-Δ::*KanMX4*) encoding a subunit of N-terminal acetylase. The *bars* showing the relative activities represent the mean calculated from 12 measurements (triplicates each with four independent extracts). Standard deviations are depicted *above the bars*. *D*, levels of the β7/Pre4 (*top*) and α7/Pre10 (*bottom*) in the same strains (*a–d*) shown in *A–C*. *wt**, strain expressing Pre10-ha. Immunoblotting conditions were as for Fig. 1*E*.

The β7/Pre4 C-terminal Tail Is Essential for β1/Pre3-mediated Post-acidic Peptidase Activity—Previous work by others (23) had shown that a mutation in the *PRE4* gene that led to a C-terminal shortening of β7/Pre4 by 15 residues resulted in a loss of the post-acidic peptidase activity, which is mediated by the β1/Pre3 subunit. Consistent with that report, we observed a significant accumulation of β1/Pre3 precursor forms in assembled proteasomes in our mutant *pre4*-Δ*C19* (Fig. 3) together with a complete loss of post-acidic activity (Fig. 4*A*). We therefore asked whether the defect in post-acidic activity in this mutant was due to a defect in β1/Pre3 processing. To answer this question, we generated a congenic set of strains carrying on one side either the *pre4*-Δ*C19* allele or its wild-type counterpart, and on the other side a gene expressing a mature form of β1/Pre3 (Pre3-Δpro) or wild-type Pre3. The β1/Pre3 propeptide is dispensable for proteasome assembly *in vivo* (20, 24), but its deletion inactivates the post-acidic activity since its unprotected N terminus is subject to Nα-acetylation (20). Inactivation of the *NAT1* gene encoding a subunit of Nat1-Ard1 Nα-acetyltransferase, however, restores near wild-type levels of post-acidic activity to the *pre3*-Δ*pro* strain (20). Therefore all strains used in the experiment shown in Fig. 4 in addition lacked the *NAT1* gene. As shown in Fig. 4*D*, none of the mutations affected the stability of β7/Pre4 as its ratio to subunit α7/Pre10 was not affected. Indistinguishably from the *pre4*-Δ*C19* single mutant, the *pre4*-Δ*C19 pre3*-Δ*pro* double mutant did not display any post-acidic peptidase activity (Fig. 4*A*). In extracts of the *pre3*-Δ*pro* single mutant, in contrast, wild-type activity levels were detected (Fig. 4*A*). We conclude that the C-terminal extension of β7/Pre4 is essential for post-acidic activity of the proteasome independent of an apparent role in mediating efficient autocatalytic processing of Pro-β1/Pro-Pre3.

In contrast to its effect on post-acidic activity, the *pre4*-Δ*C19* mutation resulted only in a slight reduction of proteasomal chymotryptic and tryptic activity (Fig. 4, *B* and *C*). These data suggest that the C-terminal extension of β7/Pre4, aside of its role in the dimerization of half-proteasome precursors, has a specific role in the formation of the post-acidic peptidase site. Consistent with previous studies (8, 9, 24), the post-acidic activity does not appear to be required for the activation of the chymotryptic and tryptic peptidase sites.

Dissection of the Role of the β7/Pre4 C-terminal Extension in Post-acidic Activity—To further understand the involvement of the C-terminal extension of β7/Pre4 in the activity of the post-acidic peptidase site, we prepared a set of mutants expressing β7/Pre4 variants with truncated C termini (Fig. 5). We asked in particular whether truncations that included the residues Ile^{225} and Gly^{219} affected post-acidic activity of the proteasome. These two residues are involved in a network of hydrogen bonds formed with residues flanking the Lys^{33} in the catalytic center of β1/Pre3 (see above; Fig. 1*B*). A deletion of only the last amino acid residue Ile^{225} (*pre4*-Δ*C1*) resulted in a drop of post-acidic activity in crude extracts by 20% in comparison with the wild type consistent with a significant role of this residue in the post-acidic activity (Fig. 5*A*). A further deletion into the C terminus that included residue Gly^{219} resulted in a loss of about 90% of the post-acidic activity. None of the deletions affected the stability of the β7/Pre4 subunit as its ratio to α7/Pre10 was unchanged (Fig. 5*B*). Taken together these data are consistent with a model (depicted in Fig. 1*B*) in which the C-terminal extension of β7/Pre4 engages in hydrogen bonds that are essential to stabilize an active conformation of the catalytic site in β1/Pre3. This function of the β7/Pre4 C-terminal extension can be separated from its role in half-proteasome precursor dimerization (see below).

Activation of the Post-acidic Site and Its Role in Proteasome Assembly Are Not Correlating Functions of the β7/Pre4 Tail—Because the deletion of only seven residues of the β7/Pre4 tail resulted in a drop of the post-acidic activity to less than 10%, we asked whether the formation or stability of the assembled 20 S proteasome was also affected by this shorter tail truncation. To answer this question, we analyzed the distribution of an epitope-tagged version of β2/Pup1 (Pup1-ha) in crude extracts of *pre4*-Δ*C7* mutant cells fractionated by gel filtration. In this mutant, the amount of precursor complexes containing unprocessed proPup1-ha was far less strikingly increased than in the *pre4*-Δ*C19* mutant (Fig. 2). Only a slight increase of such precursor complexes was detected in *pre4*-Δ*C7* in comparison to wild type. We conclude that a truncation of the β7/Pre4 C-terminal extension that nearly abolishes post-acidic activity only moderately affects proteasome assembly supporting a model assigning two distinct functions to this domain.

Truncation of β7/Pre4 C Terminus and Deletion of UMP1 Result in a Synthetic Growth Defect—Despite the observed defects in proteasome assembly (see above), the *pre4*-Δ*C19* mutant cells showed no significant growth defects at 30 or 37 °C when compared with congenic wild-type cells (Fig. 1*D* and data not shown). This observation is in accordance with the finding that the overall amount of active proteasomes in the cell, as judged by the chymotryptic and tryptic activities in this mutant, was very similar to that of wild type (see above). These data suggested that the reduced efficiency of assembly of 20 S

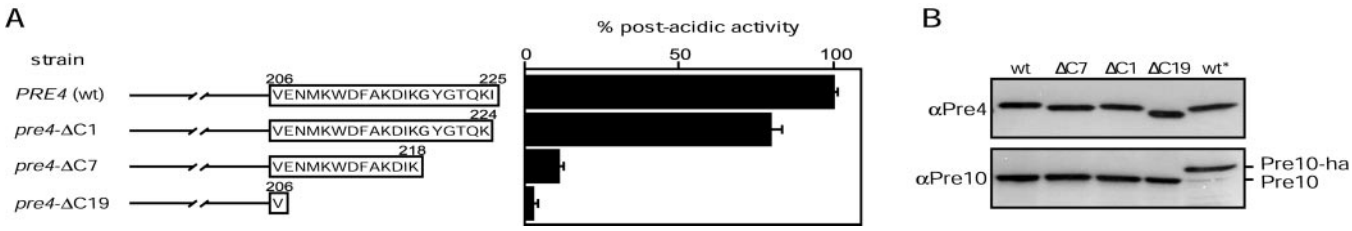

FIG. 5. **Effects of C-terminal truncations of β7/Pre4 on post-acidic peptidase activity.** *A*, shown are the relative proteasomal post-acidic activities in crude extracts from cells with wild-type (*wt*) Pre4 (set to 100%) or from strains with truncated Pre4 shortened at the C terminus by one (Δ*C1*), seven (Δ*C7*), or 19 residues (Δ*C19*). The strains used were, respectively, JD284-6D, JD310, JD311, and JD300-3D (Table I). The *bars* represent the mean of nine measurements (triplicates on three independent extracts each). Standard deviations are indicated. *B*, β7/Pre4 (*top*) and α7/Pre10 (*bottom*) levels in crude extracts from wild-type (*wt*), *pre4*-Δ*C7*, *pre4*-Δ*C1*, and *pre4*-Δ*C19* cells, respectively. *wt**, strain expressing Pre10-ha. Immunoblotting conditions were as for Figs. 1*E* and 4*D*.

proteasomes from its precursors is compensated by a moderate increase in the expression of proteasome subunit genes. We have shown previously that maturation factor Ump1 is required for efficient assembly of 20 S proteasomes from two half-proteasome precursors (19), similar to what is described above for the C-terminal extension of β7/Pre4. To test whether the *ump1*-Δ mutation and the *pre4*-Δ*C19* mutation have a synthetic effect, we crossed strains with these two mutations and analyzed the progeny by tetrad dissections. Consistent with the observation that both mutations affect the dimerization of half-proteasome precursor complexes, the double mutant showed a strong synthetic growth defect (Fig. 1*D*). To test whether the observed synthetic growth inhibition by the *pre4*-Δ*C19* and *ump1*-Δ mutations is due to an imbalance of proteasome subunits, *e.g.* due to a more rapid turnover of the mutant Pre4-ΔC19 subunit, we compared the levels of subunits β7/Pre4 and α7/Pre10 by immunoblotting. The results shown in Fig. 1*E* demonstrate that the overall levels of these subunits are similar in the single mutants and in the double mutants supporting the notion that the synthetic phenotype is due to a strong inhibition of proteasome assembly. Similar to what we observed previously for the processing of the active subunits β1, β2, and β5 (19), the *ump1*-Δ mutation also inhibited the processing of β7/Pre4 (Fig. 1*E*, *lanes c* and *d*).

The Deletion of the β2/Pup1 C-terminal Extension Is Lethal—Another prominent feature that distinguishes eukaryotic β-subunits from those in the *Thermophilus* "Urproteasome" is a ~30-residue C-terminal extension of the β2 subunit (Fig. 6*C*). Analysis of the crystal structure of the yeast proteasome (Fig. 6*A*) revealed that the extension of β2/Pup1 wraps around the β3/Pup3 subunit within the same ring of β-subunits. To study the relevance of this extension to the biogenesis of functional proteasomes, we replaced one copy of the *PUP1* gene in a diploid yeast strain with an otherwise identical copy in which the codon for Tyr^{232} (corresponds to Tyr^{203} in the processed subunit of the known crystal structure reported in Ref. 6; *underlined* in Fig. 6*C*) was replaced by a TAA translation termination codon followed by the T_{CYC1} terminator. The resulting *pup1*-Δ*C30* allele encodes a Pup1 protein that is truncated at the C terminus by 30 residues. To test for the effect of this truncation on the physiology of *S. cerevisiae*, we analyzed tetrads of ascospores derived from the resulting heterozygous strain JD329 (Table I). As shown in Fig. 6*B*, each tetrad yielded only two viable spore clones, all of which were tryptophan-auxotrophic, indicating that they lacked the *TRP1*-marked *pup1*-Δ*C30* allele. Microscopic inspection of the inviable spore clones revealed that the spores had germinated and undergone a few cell divisions before they stopped dividing (data not shown). The tetrad analysis therefore demonstrated that the C-terminal extension of β2 is essential for viability of *S. cerevisiae* cells. We conclude that this extension is essential for the generation of functional proteasomes. To investigate whether the Pup1-ΔC30 subunit is capable of assembling into proteasomal complexes and to follow the fate of the resulting complexes, we generated a diploid strain that expressed an epitope-tagged version of the truncated Pup1 (Pup1-ΔC30-ha). Extracts from the resulting strain JD325 (see Table I) were analyzed by gel filtration and immunoblotting and compared with an otherwise identical wild-type strain expressing Pup1-ha. As shown in Fig. 6*D*, unprocessed Pup1-ΔC30-ha was detectable in increased amounts in fractions 22 and 23 that typically contain the Ump1-containing half-proteasome precursor complex (19) and moreover was also present in the fractions 19–21 comprising larger assemblies similar in size to the 20 S particles. When compared with a wild-type strain, however, only minute quantities of processed Pup1-ΔC30-ha were detectable in fractions 15–19 that typically contain 26 S proteasome ($CP{\cdot}R_2$) and the 20 S particle (CP) with one regulatory particle (CP·R)(Fig. 6*D*). Similarly, we observed a striking reduction of Pup1-ΔC30-ha-containing CP·R or $CP{\cdot}R_2$ complexes when we analyzed crude extracts from the mutant strain by native PAGE (Fig. 6*E*). Note that the experiments with the Pup1-ΔC30-ha variant were performed with a diploid strain carrying also a wild-type *PUP1* gene copy. The small amounts of larger assemblies containing processed forms of Pup1-ΔC30-ha therefore may depend on the presence of a functional Pup1 subunit in the other half of the complex. We conclude that Pup1-ΔC30 assembles into proteasomal complexes but that formation of 26 S proteasomes containing this truncated subunit is impaired.

DISCUSSION

Several proteasomal β-subunits of eukaryotic proteasomes are distinguished from their counterparts in bacteria by striking C-terminal extensions. In this report we show that the C-terminal extension of the yeast β7/Pre4 subunit serves two distinct functions in proteasome biogenesis and activity. First, it functions as a molecular clamp mediating and stabilizing the interaction of two half-proteasome precursor complexes. The second function that can be assigned to the seven C-terminal residues of Pre4 is to keep the post-acidic site in an active conformation. Another finding of this study is that the C-terminal extension of β2/Pup1 serves an essential function in proteasome biogenesis.

The C-terminal Tail of β7/Pre4 Acts as a Molecular Clamp Stabilizing the 20 S Proteasome—The C-terminal tail of β7/Pre4, which extends from one half of the proteasome to the other (Fig. 1*A*), facilitates the formation of 20 S particles from two half-proteasome precursor complexes. In the *pre4*-Δ*C19* mutant lacking this extension, we detected an accumulation of half-proteasome precursor complexes (Figs. 2 and 3). The overall proteasomal chymotryptic or tryptic activities detected in this mutant, however, were only slightly reduced (less than 5 and 9%, respectively) when compared with wild type (Fig. 4, *B* and *C*). Similar to effects observed previously in mutants with

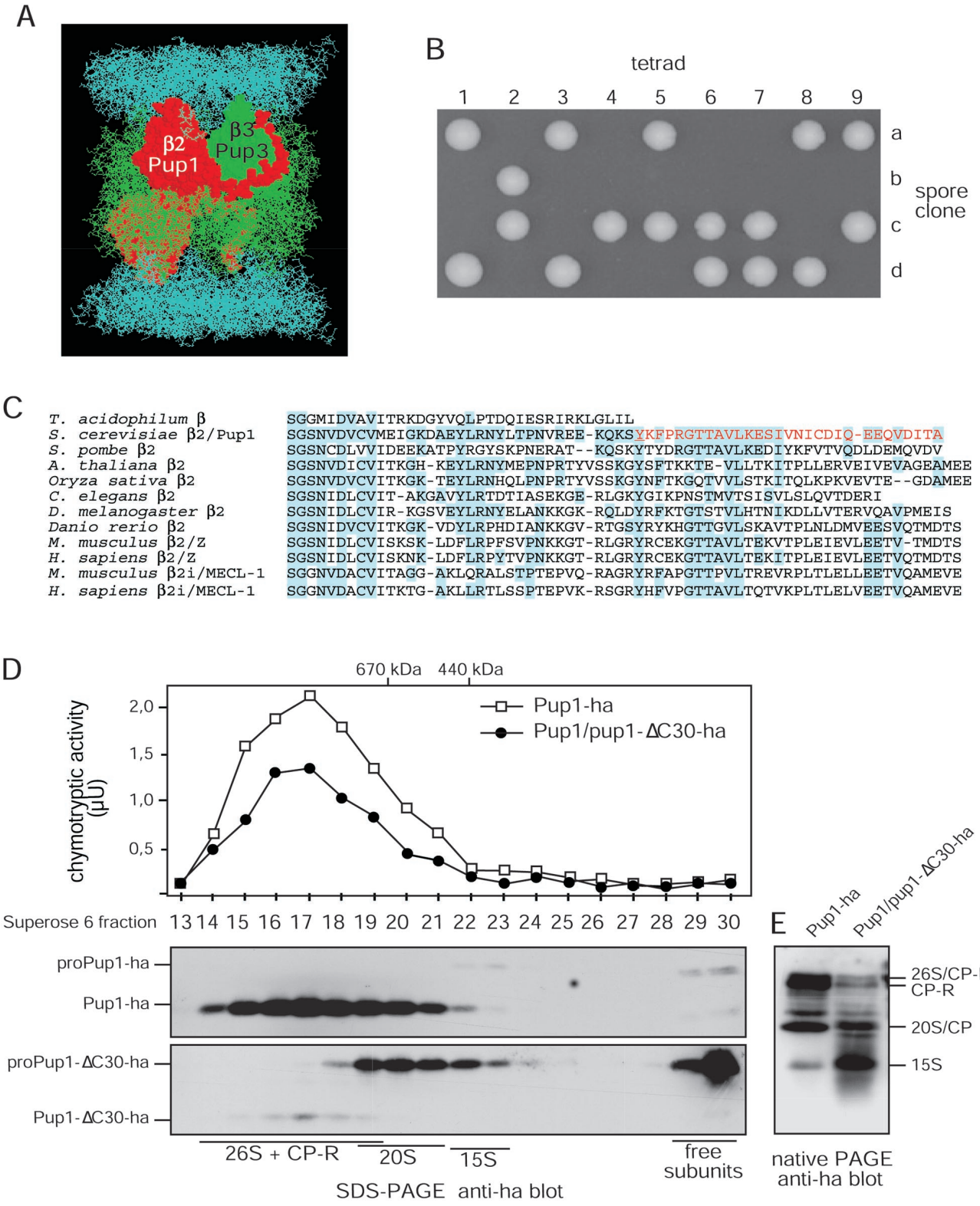

FIG. 6. **The C-terminal domain of β2/Pup1 is essential for the formation of functional proteasomes.** *A*, structure of *S. cerevisiae* 20 S proteasome (6) emphasizing the interaction of β2/Pup1 and β3/Pup3. This figure was prepared with RasMol 2.6. β2/Pup1 and β3/Pup3 are shown in the spacefill representation; all other subunits are displayed as wireframe. *B*, deletion of 30 amino acid residues from the C terminus (Δ*C30*) of β2/Pup1 is lethal. Shown is a tetrad analysis of strain JD329 (Table I) heterozygous for *pup1-ΔC30*. *C*, amino acid sequence alignments of the C-terminal regions of β2 subunits of the indicated species. Residues identical between β2/Pup1 from *S. cerevisiae* and other species are *boxed*. The C-terminal 30 residues that form the extension shown in *A* are highlighted in *red*. *D*, crude extracts of strain JD139 expressing Pup1-ha and of a heterozygous diploid strain containing one gene copy expressing the truncated β2/Pup1-ΔC30-ha (JD325; Table I) were fractionated by gel filtration. *Top*, to confirm reproducibility of the fractionations, the chymotryptic activities in the fractions obtained from both strains are compared. Thyroglobulin (670 kDa) and ferritin (440 kDa) were run on the same column. The positions of the peaks of these molecular weight standards are indicated on the *top*. *Middle* and *bottom*, the same fractions were analyzed by SDS-PAGE and immunoblotting. *E*, crude extracts from the same strains as in *D* were analyzed by native PAGE and immunoblotting.

TABLE I
Yeast strains

Strain	Relevant genotype	Source/comment
JD47-13C	MAT**a** *his3-Δ200 leu2-3, 112 lys2-801 trp1-Δ63 ura3-52*	Ref. 17
JD51	*MAT***a**/*MATα* (diploid strain isogenic to JD47-13C)	Ref. 17
JD139	*MAT***a** *PUP1-ha*::*YIplac211*	Derivative of JD47-13C
JD183	*MAT***a** *PRE10-ha*::*YIplac211*	Derivative of JD47-13C
JD234	*MAT***a** *pre4-ΔC19*::*YIplac204 PUP1-ha*::*YIplac211*	Derivative of JD47-13C
JD284-6D	*MATα nat1-Δ*::*KanMX4*	Derivative of JD47-13C
JD300-12C	*MATα nat1-Δ*::*KanMX4 UB-pre3-ΔLS*	Derivative of JD47-13C
JD300-3D	*MATα nat1-Δ*::*KanMX4 pre4-ΔC19*::*YIplac204*	Derivative of JD47-13C
JD300-5C	*MATα nat1-Δ*::*KanMX4 pre4-ΔC19*::*YIplac204 Ub-pre3-ΔLS*	Derivative of JD47-13C
JD301-3C	*MAT***a** *PRE3-ha*::*YIplac211*	Derivative of JD47-13C
JD301-5A	*MAT***a** *pre4-ΔC19*::*YIplac204 PRE3-ha*::*YIplac211*	Derivative of JD47-13C
JD310	*MATα pre4-ΔC1*::*YIplac204*	Derivative of JD284-6D
JD311	*MATα pre4-ΔC7*::*YIplac204*	Derivative of JD284-6D
JD320	*MATα pre4-ΔC7*::*YIplac204 PUP1-ha*::*YIplac211*	Derivative of JD310
JD325	*MAT***a**/*MATα PUP1 pup1-ΔC30-ha*::*YIplac211*	Derivative of JD51
JD329	*MAT***a**/*MATα PUP1 pup1-ΔC30*::*YIplac204*	Derivative of JD51
JD81-1A	*MATα ump1-Δ*::*HIS3*	Derivative of JD51
JD281	*MAT***a**/*MATα PRE4 pre4-ΔC19*::*YIplac204 UMP1 ump1-Δ*::*HIS3*	JD81-1A × JD234

proteasome defects (19), the assembly defect in the *pre4-ΔC19* mutant therefore seems to be partly compensated by a slightly increased expression of proteasome subunits (Fig. 4*D*). As a result, the *pre4-ΔC19* mutant strain does not display any obvious growth defects or phenotypes. When we combined the *pre4-ΔC19* with the *ump1-Δ* mutation, however, we observed a striking synthetic inhibition of growth (Fig. 1*D*). Similar to the data presented here for *pre4-ΔC19*, the *ump1-Δ* mutation was shown previously to impair the assembly of 20 S proteasomes from two half-proteasome precursor complexes (19).

Why do eukaryotic proteasomes require a C-terminal extension of β7 to mediate efficient assembly of proteasomes while *T. acidophilum* β-subunits lack such a feature? In this archaea, the interaction between half-proteasome precursor complexes is apparently stabilized by other features. One such feature of *T. acidophilum* β-subunits at the interface of the two β-rings could be a small extension in form of the side chain of Arg^{165}. This residue is located at the end of helix H4 that protrudes from the core of the β-subunit and is 38 residues distant from its C terminus (5). These seven small protuberances from each β-ring extend to the respective other ring into grooves between the β-subunits (5). Collectively, these zipper-like interactions may serve a similar holding function as the long C-terminal extension of β7 in eukaryotes. Despite a significant sequence divergence and a reduction in length by four amino acid residues in comparison to its *S. cerevisiae* counterpart (Fig. 1*C*), the C-terminal region of the bovine β7 subunit occupies a similar location between the β1 and β2 subunits in the crystal structure. The smaller number of hydrogen bonds that the bovine β7 engages in, however, may result in a more flexible contact allowing for an easier replacement of housekeeping subunits by interferon-induced immunosubunits β1i and β2i (11). The shorter extension of bovine β7 appears to be compensated by an additional extension of the β1 subunit (11). These two extensions of the neighboring subunits β1 and β7 seem to form a short "zipper."

The C-terminal Seven Residues of β7/Pre4 Are an Important Structural Component of the Post-acidic Peptidase Site—Previous work by others (23) had already shown that a shortening of the β7/Pre4 C terminus (*pre4-ΔC15*) results in a loss of the post-acidic peptidase activity mediated by the β1/Pre3 subunit. Consistent with these findings, we observed almost a total loss of post-acidic peptidase activity in our *pre4-ΔC19* mutant (Fig. 4*A*). Because we also observed a defect in the processing of proβ1/Pre3 (Fig. 3) in this strain, we asked whether the C-terminal extension of β7/Pre4 is important to trigger autocatalytic processing of the propeptide of β1/Pre3 or whether it is an essential structural component of the post-acidic peptidase active site *per se*. That the latter is true was concluded from the lack of post-acidic activity in a strain expressing a mature form of β1/Pre3 (Ub-Pre3-Δpro in Fig. 4) and β7/Pre4 lacking its C-terminal extension. We conclude that the β7/Pre4 C-terminal extension is an essential component of the post-acidic site by providing a network of hydrogen bonds that stabilizes an active conformation of β1/Pre3 as illustrated in Fig. 1*B*. Although one of the shorter truncations removing only seven residues (*pre4-ΔC7*) resulted in a severe reduction in post-acidic activity (by ~90%; Fig. 5), it had relatively little effect on the dimerization of two half-proteasome precursor complexes (Fig. 2). We interpret this result as evidence that these two functions of the C-terminal extension of β7/Pre4 are distinct and separable. A comparison of the C-terminal extensions of the orthologous β7 subunits of *S. cerevisiae* and other species revealed that it is not highly conserved in sequence (Fig. 1*C*). This observation suggests that this domain in the *S. cerevisiae* β7/Pre4 is specifically adapted to its function in post-acidic activity in the context of the budding yeast 20 S proteasome.

The C-terminal Extension of β2/Pup1 Is Essential for Proteasome Biogenesis—In comparison with β-subunits of the *T. acidophilum* proteasome, subunit β2 of eukaryotic proteasomes is characterized by a C-terminal extension of ~30 amino acid residues (Fig. 6*C*). As shown in Fig. 6*A*, this extension wraps around the neighboring subunit β3/Pup3 in the same β-ring of the *S. cerevisiae* proteasome (6). A similar structural arrangement was found for the subunits β2 and β3 in the bovine proteasome (11). We show that a deletion of this extension is lethal to yeast cells (Fig. 6*B*). Our experiments with a diploid strain that expressed wild-type β2/Pup1 in addition to an epitope-tagged variant of the truncated Pup1 (*pup1-ΔC30-ha*) showed that the latter is incorporated into proteasomal complexes. Propeptide processing of the Pup1-ΔC30-ha variant and the formation of 26 S proteasomes containing this subunit, however, were very inefficient (Fig. 6). One possible explanation is that subunit β3/Pup3, which seems to be held in place by the β2/Pup1 C-terminal tail, cannot be incorporated properly in the absence of this tail leading to an inhibition of the assembly and maturation of 26 S proteasomes.

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Role of C-terminal Extensions of Subunits β2 and β7 in Assembly and Activity of Eukaryotic Proteasomes

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