The α4 and α7 subunits and assembly of the 20S proteasome

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Abstract The detailed mechanism of eukaryotic 20S proteasome assembly is currently unknown. In the present study, we demonstrate that the 20S proteasome subunits α4 and α7 interact with each other as well as all the α-subunits in vivo and in vitro. The N-terminal parts of α4 and α7 are essential for these newly discovered interactions in vitro. Glycerol gradient centrifugation of soluble extracts of HEK293 cells and Western blot analyses show that several α-subunits are found in non-proteasomal low-density fractions. The α4 and α7 subunits co-immunoprecipitate together from these low-density fractions. The unexpected interaction between α4 and α7 may provide a molecular basis for the formation of previously reported 13S and 16S assembly intermediates.

Keywords: 20S proteasome; α-subunit; Two-hybrid system; In vitro translation; α-ring assembly

1. Introduction

The eukaryotic 20S proteasome has a complex molecular architecture, which requires the co-ordinated assembly of a large number of different subunits.

The 26S proteasome consists of the central 20S proteasomal core, generally capped on both ends by a 19S regulator, which can recognise and degrade ubiquitin-protein conjugates in an ATP-dependent manner [1]. Some eukaryotic cells also contain another proteolytic particle termed the 20S–11S complex. This complex again consists of the central 20S core, but is capped by an alternative regulator known as 11S (PA28), which is implicated in antigen processing [2]. This complex has been crystallised, revealing that seven tail-like projections of the 11S regulator bind equally to pockets located between the α-subunits of the 20S core and cause conformational changes in those subunits [3].

The assembly of the α-subunit ring is critical in the biogenesis of eukaryotic proteasomes, however the mechanism of α-ring assembly has yet to be elucidated. Structural intermediates have been reported with sedimentation coefficients of approximately 13S and 16S [4,5]; the 13S particle contains seven α-subunits and three unprocessed β-subunits [4], while the rapid dimerisation of two half-proteasomes, each composed of an α and β-ring, creates a population of 16S complexes [6]. Within these intermediate complexes, β-subunit propeptides are removed by limited proteolysis [7], converting inactive assembly intermediates into mature 20S proteasomes [8]. The maturation of the yeast 20S proteasome also requires a 16.8 kDa chaperone called Ump1 [9]. This protein is a component of a precursor complex which sediments at ~15S, but is not detected in the mature 20S proteasome since Ump1 is degraded during the assembly of the particle. Recently, a human homologue of Ump1, designated POMP, has been found. This protein is associated with the 16S precursor but not with the 20S proteasome [10].

Although proteasomal α- and β-subunits have similar three dimensional structures [3], the uniqueness of individual α-subunits resides in the conservation of additional N-terminal extensions as well as differences in their C-termini. The N-terminal extensions, approximately 35 residues in length, each form a helix, which together seal the central channel in the crystal structure of the 20S particle and regulate substrate access into the catalytic core [11,12]. Furthermore, it has been demonstrated in yeast that the N-terminal segments of α1, α2, α3 and α7 are essential for contact between the α-subunits within the α-ring [12]. Moreover, when expressed in *Escherichia coli*, some recombinant eukaryotic α-subunits including the human α7 (HsC8) and *Trypanosoma brucei* α5 (ζ-subunit) self assemble into double and even four stacked protein rings [13,14]. Similarly, two other α-subunits HsPROs 27 (α1) and HsPROs 30 (α6) can also form protein dimers [15]. In addition, human α7 protein can induce hetero-oligomeric ring formation when co-expressed with α1 and α6 subunits, which are adjacent to α7 in each α-subunit ring of 20S proteasome [15].

In this study, we have used the yeast two-hybrid (Y2H) screen and biochemical analyses to study putative interactions between all seven human α-subunits to ascertain whether protein interactions, in addition to those seen in the crystal structures of the 20S proteasome, may occur. Such interactions may determine α-ring assembly and perhaps precede those precipitated by β-subunits or stabilised by molecular chaperones (e.g., hsc73) or transient facilitatory molecules (e.g., POMP) proteins during the overall program of hemiproteasome biogenesis.

2. Materials and methods

2.1. Preparation of expression vectors

The seven human alpha subunit cDNAs were amplified by RT-PCR and cloned in frame into the following vectors pET41 (Novagen), pCITE (Novagen), pGBT9 and pGAD GH (Clontech), generating

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constructs pET41-α_{1-7}, pCITE-α_{1-7}, pGBT9-α_{1-7} and pGAD GH-α_{1-7}. All constructs used in the study were sequenced to confirm identity and PCR fidelity.

2.2. Deletional mutagenesis of α4 and α7

Deletion mutagenesis was carried out by PCR or restriction digestion using either α4 or α7 DNA templates. The truncated forms of α4 are α4ΔN1 (1–40 amino acids) and α4ΔC1 (80–248 amino acids). The truncated forms of α7 are α7ΔN1 (1–100 amino acids) and α7ΔC1 (134–255 amino acids).

2.3. The yeast two-hybrid system

The two-hybrid method is a yeast-based genetic assay which has been previously described [16].

Two yeast strains carrying the auxotrophic markers *trp*1 and *leu*2 were used for all the Y2H work. Strain HF7c has a *lac*Z reporter gene under the control of the artificial upstream activating sequence $UAS_{G17\text{-}mer\ (x3)}$ and a *HIS*3 reporter gene under the control of an intact *GAL*1 UAS. Strain SFY526 has only a *lac*Z reporter gene, but it is controlled by an intact *GAL*1 UAS and therefore can be expressed at ~10× the level obtained with the $UAS_{G17\text{-}mer\ (x3)}$ promoter. For qualitative analysis of Y2H interactions, activity of the β-galactosidase reporter gene was assessed as described previously [17].

2.4. Expression, purification and binding analyses of recombinant proteins

Transformed *E. coli* strain BL21 DE3 (Stratagene) was grown at 37 °C in LB medium containing Kanamycin (50 μg/ml) until logarithmic phase (OD_{600} at 0.6) before induction with 1 mM IPTG for 1 h. Cells were harvested by centrifugation, resuspended in PBS buffer containing 1% (v/v) Tween 20 and disrupted by a French press. GST or GST-α-subunit containing protein extracts were incubated with glutathione (GSH)-Sepharose beads (Amersham Pharmacia Biotech) and purified according to the manufacturer's protocols.

For in vitro transcription/translation, the single tube protein system 3 (STP3) kit (Novagen) was used according to the manufacturer's instructions.

For binding studies with radiolabelled subunits, equal amounts (9 μg) of GST or GST-α-subunit were immobilised onto (GSH)-Sepharose beads and processed as described [17]. Protein fractions were subjected to SDS–PAGE followed by Western blot analyses, Coomassie staining or phosphor-imager analyses (Fujifilm FLA-2000R).

2.5. Cell culture and reagents

Human HEK293 cells were cultured under standard conditions in DMEM containing 10% foetal calf serum.

Antibodies against proteasomal subunits were kindly provided by Affiniti Research Products, Exeter, England.

2.6. Glycerol gradient fractionation, Western blot analyses and immunoprecipitation

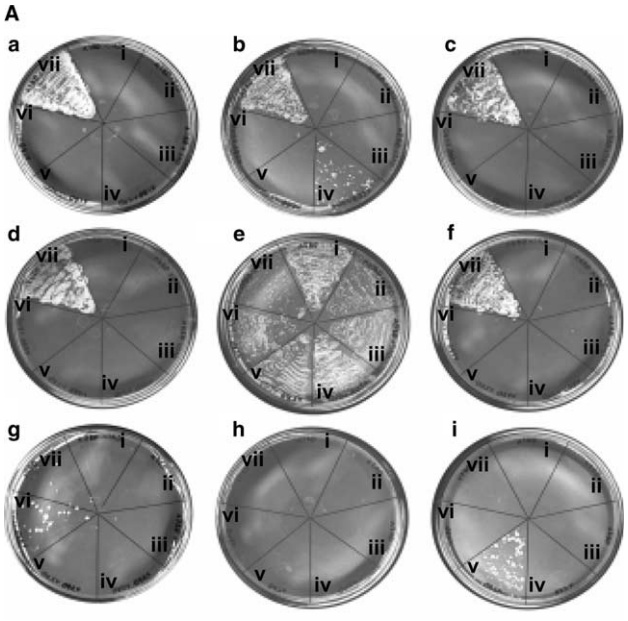
Cell extracts were subjected to velocity gradient ultracentrifugation as described previously [18]. Each fraction (30 μl) was analysed by SDS–PAGE and immunoblotting. An identical gradient employing density gradient marker proteins was centrifuged simultaneously so that the sedimentation coefficients of proteins in the fractions could be determined. Each fraction (30 μl) was also subjected to dot-blotting and staining with Coomassie brilliant blue so that the fractions in which proteins were localised could be easily determined.

Equal amounts of protein were subjected to immunoprecipitation and processed according to a previously described protocol [19]. The precipitates were subjected to SDS–PAGE, transferred onto nitrocellulose membranes by electroblotting and immunodetected as described [10].

3. Results

3.1. α-Subunit interactions

The crystal structure of the 20S proteasome reveals that intimate contacts are made by the intertwined N-terminal segments of subunits α1, α2, α3 and α7 [12]. Interactions between α7 and α6 and between α7 and α1 have also been reported [13]. We used a Y2H screen to identify novel α-subunit interactions, which are not predicted from the crystal structures of the 20S proteasome. The initial library screen of over two million clones with the α4 subunit surprisingly gave 23 positive interactions with the α7 subunit and no other interactions of α4 were detected. Since α4 and α7 are at opposite localisations in the α-subunit ring [12], this interaction was least expected. It is reasonable to suggest that α4/α7 interactions may have precursor functions, including in the formation of the α-ring. The α4 subunit also interacts with α2 and α6 interacts with α7. Furthermore, we have identified interactions between the α7 subunit (HC8) of the 20S α-ring, and the α1, α2, α3, α4, α6 and α7 subunits (Fig. 1A, plates a, b, c, d and f). Notably, a strong interaction was seen between the α7 and α4



B

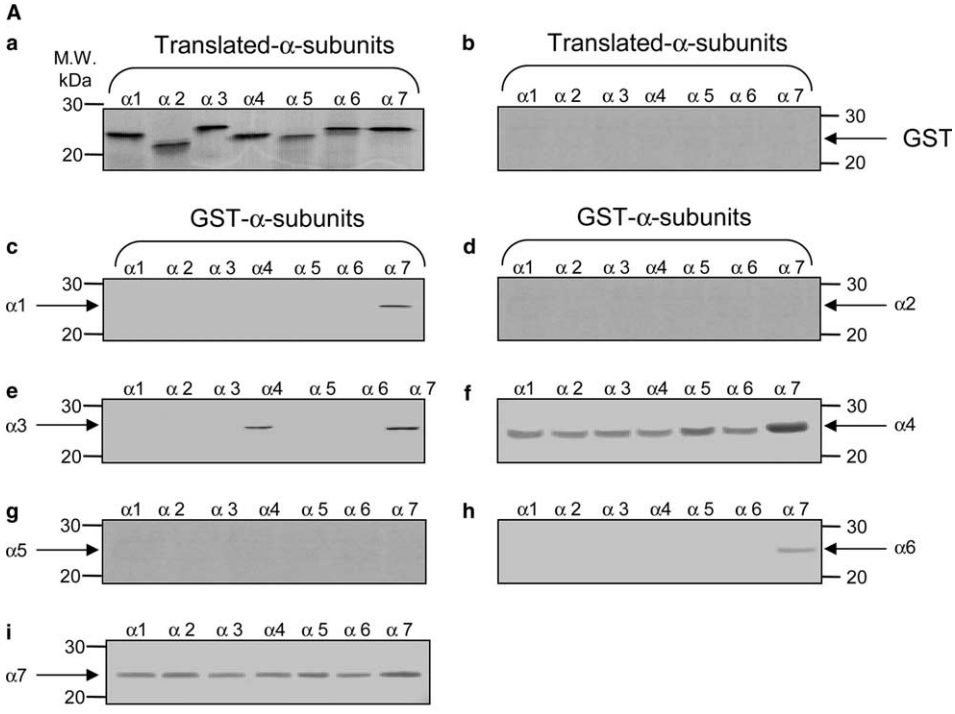
AD / BD	α1	α2	α3	α4	α5	α6	α7
α1							▨
α2				▨			▨
α3							▨
α4							▨
α5							
α6							▨
α7						▨	▨

Fig. 1. Y2H analyses of pairwise α-subunit interactions. (A) Panels a–g show the growth of co-transformants on SD-LWH (single letter amino acid code), panel h shows the growth of individual transformants on SD-LH and panel i shows the growth of individual transformants on SD-WH media. (B) Interaction map found by two-hybrid analysis. AD = activating domain fusions; BD = binding domain fusions. Hatched squares represent positive interactions.

subunits (Fig. 1A, plate d). When cloned into the DNA binding domain, the human α5 subunit transactivates the GAL4 reporter gene (Fig. 1A, plate i), rendering any α5 interactions in the Y2H screen inconclusive. This transactivation is possibly due to the acidic nature of this subunit. A similar phenomenon has previously been reported for α4 [20]. For clarity, the interactions are summarised diagramatically in Fig. 1B.

To extend and verify the interactions found by the Y2H screen, the individual α-subunits were tested for pairwise interactions in vitro (Fig. 2A and B). All the α-subunits were cloned into an expression vector and translated in the presence of [^{35}S]-methionine (Fig. 2A, panel a). Interactions between α-subunits were identified by incubating in vitro radiolabelled α-subunits with either immobilised GST α-subunits (Fig. 2A, panels c–i), or with immobilised GST alone as a control (Fig. 2A, panel b). Fig. 2A, panel i shows that in vitro translated α7 binds the six other GST-α subunits and itself, confirming the result of the Y2H screen (Fig. 1A, panels a–g). Similar binding was also shown between in vitro translated α4, the six other GST-α subunits and again itself (Fig. 2A, panel f). Notably, the binding between α4 and α7 appeared strongest (Fig. 2A, panel f, lane 7), confirming the strong interaction found in the Y2H analysis (Fig. 1A, panel d). Interestingly, α5 interacts with α4 and α7 (Fig. 2A, panels f and i) in this analysis.

We also identified interactions between in vitro translated α6 and immobilised α7 (Fig. 2A, panel h, lane 7 and panel i, lane 6), and between in vitro translated α1 and immobilised α7 (Fig. 2A, panel c, lane 7 and panel i, lane 1). These results corroborate previous findings where interactions between α7 and its naturally neighbouring subunits, α1 and α6 have been reported [13]. We also show that in vitro translated α3 interacts with immobilised α4 and α7 (Fig. 2A, panel e, lanes 4 and 7),



B

GST \ ^{35}S	α1	α2	α3	α4	α5	α6	α7
α1				▨			▨
α2				▨			▨
α3				▨			▨
α4			▨	▨			▨
α5				▨			▨
α6				▨			▨
α7	▨		▨	▨		▨	▨

Fig. 2. (A) In vitro α-subunit interactions. In vitro translated [^{35}S]-radiolabelled α-subunits (a). The binding of in vitro translated [^{35}S]-radiolabelled α-subunits to immobilised GST (b) or each GST-α-subunit (c–i). The positions of relevant molecular weight markers are shown. (B) 20S proteasomal α-subunit interaction map from data in Fig. 2A. Hatched squares represent positive interactions.

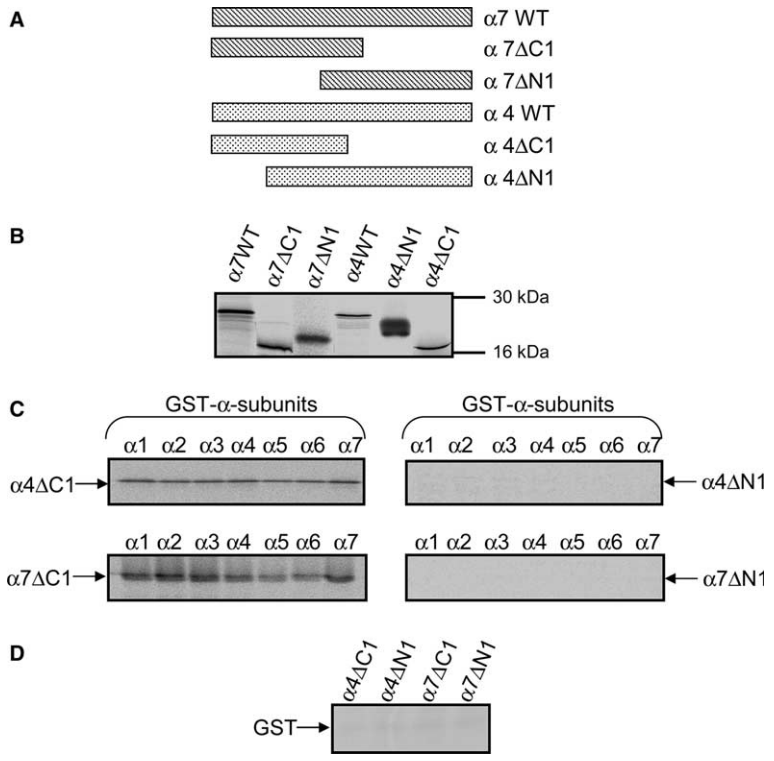


Fig. 3. The N-terminus of α4 and α7 is essential for the interaction with the other α-subunits. (A) Diagram of the wild type and deleted α4 and α7-subunits. (B) In vitro translated [^{35}S]-radiolabelled wild type and deleted α4 and α7 subunits. (C, D) The binding of in vitro translated [^{35}S]-radiolabelled deleted α4 and α7 to immobilised GST-α-subunits (C) or GST (D).

however no binding of in vitro translated α2 or α5 to any of the other six GST-α-subunits was observed (Fig. 2A, panels d and g). No interactions were detected with the GST control (Fig. 2A, panel b). Overall, we detected 18 interactions between the 20S proteasomal α-subunits which are summarised in the matrix diagram (Fig. 2B).

Curiously, the interactions identified by the two different experimental techniques are not the same. In addition, reciprocal activation in the Y2H system was not always observed. For example, an interaction was demonstrated between α1 and α7 when fused to the GAL4 DNA-binding and activation domains, respectively. However, when α7 was fused to the DNA-binding domain and α1 to the activation domain of GAL4, no reporter gene activity was detected (Fig. 1A, panels a and g). A definitive explanation for this anomaly is unknown. One plausible reason is that α-subunit interactions may be ablated by inappropriate folding of the subunits evoked by the N-terminal fusions, eliminating the site of interaction. Another explanation could be that the GAL4 and GST domains may be directly responsible for masking interaction sites. In the case of the Y2H screen, a further possibility is that the DNA-binding and activation domains are not always brought into close enough proximity to enable restoration of transcriptional activation.

3.2. The N-terminal regions of α4 and α7 are essential for interactions with other α-subunits

These findings provide evidence that α4 and α7 may be significant players in the construction of an α-ring, since α4 and α7 do not interact in the complete 20S particle [12]. In order to determine which regions of α4 and α7 are involved in interactions with other proteins, a series of deletion mutants were created for both α4 and α7 subunits (Fig. 3A). The cDNAs for the truncated proteins were cloned into the expression vector and again translated in vitro in the presence of [^{35}S]-methionine (Fig. 3B). Employing the same approach as before, we found that in vitro translated α4ΔC1 still interacted with all α-subunits (Fig. 3C), whereas α4ΔN1 did not (Fig. 3C). In addition we found that α7ΔC1 still bound all α-subunits (Fig. 3C), whereas α7ΔN1 did not (Fig. 3C). No interaction was demonstrated between GST alone and any translated mutant subunits (Fig. 3D). These results provide evidence that the N-terminal domains of α4 and α7 are critical for their interaction with each α-subunit.

3.3. Analysis of the distribution of α-subunits after glycerol gradient centrifugation of soluble extracts of HEK 293 cells

Soluble cellular proteins from HEK 293 cells were subjected to glycerol gradient centrifugation and fractions were analysed by immunoblotting for the seven α-subunits of the 20S proteasome. Western blot analysis with monoclonal antibodies raised against each of the seven α-subunits shows a different distribution for each subunit of the α-ring on the gradient. The α4, α5, α6 and α7 subunits are found in fractions 1–15 (Fig. 4), whereas α1 is found in fractions 5–15 (Fig. 5) and α2 and α3 are distributed between fractions 8 and 11 corresponding to the position of the mature 20S proteasome. The distribution of some of the seven α-subunits in earlier fractions of the gradient suggests the presence of some pre-13S precursor complexes between 4.2S and 8.9S. We observed several bands in each

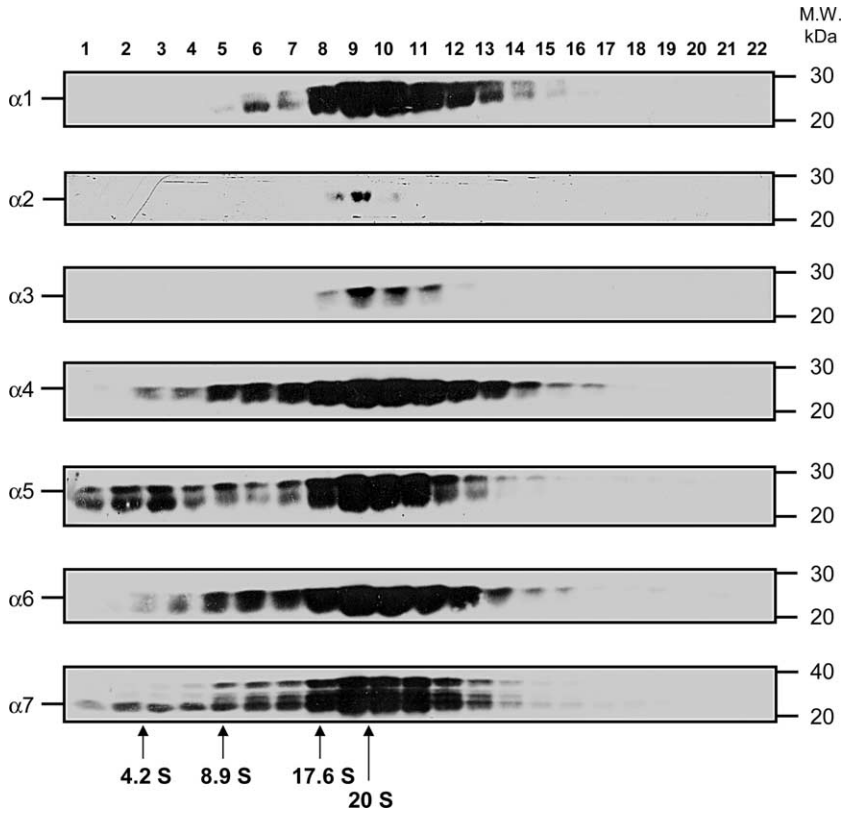


Fig. 4. Distribution of the α-subunits in soluble extracts of HEK 293 cells. HEK 293 cell extracts were sedimented on a 10–40% (w/v) glycerol density gradient. Fractions were subjected to SDS–PAGE analysis followed by immunostaining with antibodies raised against each α-subunit. Sedimentation coefficients were obtained from a gradient centrifuged in the same rotor containing proteins of known Svedberg values (proteasome: 20S, apoferritin: 17.6S, β-amylase: 8.9S and bovine serum albumin: 4.2S).

fraction when immunoblotted with the anti-α7 antibody (Fig. 4). This is probably due to α7 subunit phosphorylation as described previously [21]. Phosphorylation may also account for multiple bands observed for other α-subunits in gradient fractions.

For direct proof of the existence of pre-13S precursors in HEK 293 cells, immunoprecipitation experiments were carried out with monoclonal anti-α4 antibody on gradient fractions 1–6. The proteins co-immunoprecipitated from these fractions were further analysed by immunoblotting using monoclonal antibodies raised against the six other α-subunits. Immunoblot analysis of α4-precipitated proteins revealed bands of approximately 27 and 28 kDa predominately in fractions 5 and 6 corresponding to the α7 subunit (Fig. 5). No co-immunoprecipitated proteins were found in Western blot analyses with the five other monoclonal antibodies raised against α1, α2, α3, α5 and α6 (data not shown). Immunoprecipitation with monoclonal anti-α7 subunit was attempted, but this antibody does not immunoprecipitate efficiently enough to interpret the data. The fact that α5 and α6 subunits are detected in some fractions containing the α4-precipitated proteins may confirm the suggestion that the α5 and α6 subunits exist as monomeric forms [13,22]. Together, the data show that in addition to the 13S precursor complex, there are smaller complex(es) with S values between 4.2S and 8.9S that are composed of at least the α4 and α7 subunits.

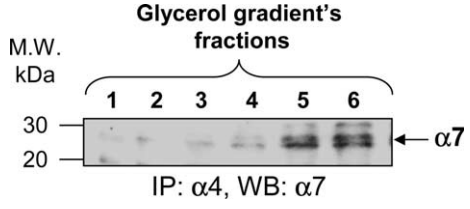


Fig. 5. Co-immunoprecipitation of α4 and α7 subunits. Immunoprecipitation (IP) with monoclonal anti-α4 antibody of proteins from gradient fractions 1–6 from HEK 293 cells. Precipitated proteins were separated by SDS–PAGE and analysed by Western blotting with anti-α-subunits.

4. Discussion

The objective of this work was to investigate the interactions of proteasomal α-subunits that might be involved in α-ring assembly. The interactions were studied by: (a) the Y2H screens, (b) in vitro binding assays with in vitro translated α-subunits and immobilised recombinant α-subunits, (c) mutagenesis analyses and (d) gradient analyses of soluble cell proteins. These approaches have led to the identification of some α–α-subunits interactions that are not predicted from the crystal structure of the yeast 20S proteasome [12] and reveal new functional interactions between subunits in the α-ring.

The work has demonstrated important novel interactions for the α4 and α7 subunits. The in vitro translated α4 and α7 subunits bind to all six GST-α-subunits immobilized on GSH-Sepharose and to themselves (Fig. 2A and B). These interactions corroborate and extend those observed in the Y2H system (Fig. 1A and B). A preliminary description of α4/α7 interactions in Y2H screens has been reported [23]. We have also shown that translated α6 and α1 bind to α7 (Fig. 2A, panels c and h) to confirm previous work [13], that α3 interacts with α4 and α7 (Fig. 2A, panel e), and that α4 interacts with α2 as shown previously in a two-hybrid study [24]. The importance of the α4 subunit in α-ring assembly is supported by the recent demonstration that α4 can substitute for the α3 subunit in the 20S proteasomal ring in yeast [25].

The assembly of the 20S proteasome has been studied in *Thermoplasma acidophilum* and other organisms, such as yeast and mammalian cells [5,12,26]. In *Thermoplasma*, it has been shown that the α-subunits assemble spontaneously into α-rings. This event is likely to be an initial step in proteasome assembly. The N-terminal parts of the α-subunits, highly conserved in all organisms, are essential for the formation of the 20S proteasome α-ring [12,27] as these regions of the proteins are necessary for interactions of translated α4 and α7 (Fig. 3).

Bacterial expression of recombinant human α7 results in the formation of a double heptameric ring structure of 540 kDa. However, the expression of human α1 or α6 subunits results in the formation of dimers only [15]. It has also been shown that the expression of *Trypanosoma brucei* α5 subunit results in the formation of a complex of four heptameric rings [14].

Gradient analysis of soluble extracts of cells shows that the α4, α5, α6, and α7 subunits are in fractions containing proteins of less than 13S. Immunoprecipitation with anti-α4 antibody co-precipitates α7 (Fig. 5) but not the other α-subunits. Based on the Y2H screen and interactions of the translated α4 and α7 subunits, it appears that these two α-subunits may form a heterodimeric precursor platform on which to build the complete α-ring.

The crystal structures of yeast and mammalian proteasomes show no interaction between the α4 and α7 subunits [12,28]. Indeed, the subunits are diametrically opposed in the crystal structure of the α-ring. However, the fact that in the Y2H screen and in vitro the α7 subunit interacts with all the other six α-subunits and the α4 subunit interacts with all six α-subunits in vitro suggests that the α4 and α7 subunits play a pivotal role in the early steps of a α-ring assembly.

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