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High yield bacterial expression and purification of active recombinant PA28 $\alpha\beta$ complex

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Running title: expression of recombinant PA28 $\alpha\beta$

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Abstract

The PA28 complexes (also termed REG or 11S complexes) are described as activators of the 20S proteasome, a major intracellular protease in eukaryotic cells. They bind to the ends of the barrel-shaped 20S proteasome, and activate its peptidase activities. The interferon γ inducible PA28 $\alpha\beta$, made of the two related subunits PA28 α and β , is under sustained investigation as it plays important roles in the production by the proteasome of class I antigen peptides. However, *in vitro* studies of this complex have been impaired by the difficulty of producing large amount of this protein, mainly due to the poor solubility of its β subunit when expressed in *E. coli*. Here we describe the construction of a bicistronic vector, allowing simultaneous production of functional human PA28 α and β subunits in *E. coli*. Co-expression of the two proteins allows efficient formation of active PA28 $\alpha\beta$ complexes, that remain soluble and can be easily purified by regular chromatographic procedures.

Introduction

The ubiquitin-proteasome system is a major intracellular proteolytic pathway that plays crucial roles in most basic cellular processes, including the cell cycle, stress responses and the regulation of immune and inflammatory responses [1, 2]. At the heart of this system is the 20S proteasome, a ~700 kDa multisubunit and multicatalytic protease highly conserved from yeast to humans, with simpler versions found in certain archaea [3, 4]. The 20S proteasome is a barrel-shaped complex, consisting of two inner rings of seven different β subunits sandwiched between two outer rings formed by seven different α subunits [5]. In eukaryotic cells, three of the seven β -subunits are proteolytically active, and define six active sites with distinct specificities when probed with artificial peptide substrates: two sites with trypsin-like activity, two sites with chymotrypsin-like activity, and two sites with caspase-like activity, due to their preferences for hydrolysis after basic, hydrophobic or acidic residues, respectively. These active sites are confined in an internal chamber formed by the two β -rings, which is accessible for substrates via an axial channel, the entry of which is controlled by the α -rings.

In native conditions, the N-terminal ends of the α -subunits form a lattice that closes the axial channel, thus impairing substrate access and maintaining the 20S proteasome in a latent form [5, 6]. However, upon binding of regulatory complexes to the 20S proteasome, rearrangement of the α -rings leads to opening of the channel, allowing substrate access to the catalytic chamber [7, 8].

One group of 20S proteasome regulatory complexes is made by the PA28 family members (also known as 11S or REG) [7, 9, 10], which are found in higher eukaryotes but are, apparently, absent from yeasts.

This family of 20S regulators is composed of three homologous ~28 kDa subunits termed α , β and γ . The α and β subunits form a heteromultimer (PA28 $\alpha\beta$), which is mainly cytoplasmic,

whereas the γ subunit forms a homomultimer (PA28 γ), which is strictly nuclear. There are conflicting data regarding the size of these multimers, as PA28 α and β have been found to form a heterohexamer (3 α 3 β) [11, 12] or a heteroheptamer (3 α 4 β) [13], whereas PA28 α forms a heptameric ring *in vitro* [7, 14].

PA28 binds to and opens the α rings of the 20S proteasome, with an alignment of the channel formed through the center of PA28 with that of the proteasome [7]. This explains how PA28 greatly stimulates peptidase activity of the 20S particle, as it enables peptide substrates to freely diffuse through the central channel of PA28 and into the proteasome interior. However, it is possible that peptide hydrolysis is further modulated by long-range conformational changes induced at the catalytic sites [15]. Although PA28 $\alpha\beta$ can bind to one or two ends of the 20S proteasome [16], it is also found in "hybrid" proteasomes in which one 20S particle is bound to one 19S regulatory complex at one end, and to one PA28 $\alpha\beta$ complex at the other end [17, 18].

Although these biochemical properties of PA28 seem to be clear, the precise roles of this complex in proteolysis remain elusive, particularly because it appears to have no stimulatory effect on degradation of proteins *in vitro*. However, evolutionary conservation implies that PA28 has important roles in higher eukaryotes and several important functions have been proposed. In particular, PA28 α and β subunits are particularly abundant in immune tissues and are induced by interferon γ and infection [10, 19], suggesting a role of PA28 $\alpha\beta$ in class I antigen production and presentation that indeed has been well documented [20-24]. However, PA28 $\alpha\beta$ also has non-immune functions, as its levels can be affected in several physiological or pathological conditions that seem to be unrelated to the immune response [25-27], including a role in HSP90-mediated protein refolding [28, 29].

Analyses of PA28 $\alpha\beta$ functions in proteolysis have been impaired by the difficulty of producing this complex *in vitro*. Besides purifying the native PA28 $\alpha\beta$ complex from cells, the

most common alternative was to express in *E. coli* and to purify the two subunits separately, before reforming the complex *in vitro*, a time-consuming and inefficient process due to the lack of solubility of the β subunit [19, 30]. As a consequence, many *in vitro* studies have been made with PA28 α alone, even though this complex probably does not exist *in vivo* [22]. To overcome this difficulty, we decided to construct a bicistronic vector allowing coexpression in *E. coli* of both PA28 α and β subunits. As expected, the two subunits spontaneously assemble into an active PA28 $\alpha\beta$ complex, that can be easily purified to apparent homogeneity by classical chromatographic procedures.

Materials and methods

Materials

Chemicals and reagents

Restriction enzymes, T4 DNA ligase and alkaline phosphatase (CIP) were purchased from Ozyme (Saint Quentin Yvelines, France), prestained Protein Marker (Broad Range, 6-175 kDa) from Biolabs (Ipswich, MA, USA), Ampicillin from Roche Diagnostics (Meylan, France) and IPTG and DTT from Euromedex (Mundolsheim, France).

The goat anti-PA28 β antibodies and the rabbit anti-PA28 α antibodies were purchased from Santa Cruz biotechnology (Heidelberg, Germany) and Biomol (Exeter, UK), respectively. The horseradish peroxidase-conjugated monkey anti-goat IgG and goat anti-rabbit IgG antibodies were purchased from Santa Cruz biotechnology (Heidelberg, Germany) and from Bio-Rad (Marnes la Coquette, France), respectively.

Western lighting Chemiluminescence reagent plus was purchased from Perkin Elmer (Paris, France).

Oligonucleotides primers (MWG, Ebersberg, Germany) for PCR amplification of PA28 α and β cDNAs were as follows:

PA28 α forward primer: 5' GCACATATCATATGGCCATGCTCAGGGTCC 3';

PA28 α reverse primer: 5' GCGGGATCCTCAATAGATCATTCCCTTTGTTTCTCCC 3';

PA28 β forward primer: 5' GCGCGATATCAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCCAAGCCGTGTGGGGTGCGCC 3';

PA28 β reverse primer 5' GCGCCTCGAGTCAGTACATAGATGGCTTTTCTTCACCC 3'.

Chromatography columns

High Q 5 mL and CHT II 5 mL were purchased from Bio-Rad (Marnes la coquette, France), Mono Q 10/10 and Superose 12 from GE Healthcare (Orsay, France). All chromatographic steps were performed using the Biological Duo Flow chromatography system (Bio-Rad).

Methods

Construction of pET-PA28 $\alpha\beta$ bicistronic plasmid

The cloning vector pET-17b (Novagen) was amplified in *E. coli* DH5 α . Human PA28 α and PA28 β cDNAs were amplified by PCR, from plasmids CD498 and CD451 (kind gift from Dr. M. Piechaczyk, IGMM, Montpellier, France), respectively. For PA28 α , the forward primer includes a NdeI restriction site (CATATG), and the reverse primer a BamHI restriction site (GGATCC). For PA28 β , the forward primer includes a EcoR V (GATATC) restriction site and a Ribosome Binding Site (AAGGAG), and the reverse primer contains a XhoI (CTCGAG) restriction site.

After amplification, PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Hoerd, France).

PA28 α PCR products and the vector pET-17b were subjected to a double digestion with NdeI and BamHI. After treatment with alkaline phosphatase (CIP) of the linearized vector, both DNA fragments were purified on 1% agarose gels and subsequently extracted from the gel using NucleoSpin Extract II kit, then ligated with T4 DNA ligase at 16°C overnight. TOP 10 competent *E. coli* (Invitrogen, Cergy Pontoise, France) were transformed with the ligation mixture, and positive clones were selected on LB agar plates containing 100 μ g/mL ampicillin. A single colony was cultured in LB media containing 100 μ g/mL ampicillin and the plasmid (pET-17b-PA28 α) was purified using the Plasmid Midi kit (Qiagen, Courtaboeuf, France).

PA28 β PCR products and plasmid pET-17b-PA28 α were subjected to a double digestion with EcoRV and XhoI. Subsequent steps were performed essentially as above and yielded the pET-PA28 $\alpha\beta$ bicistronic plasmid. The sequence of the insert was then verified using primers corresponding to the T7 promoter and terminator present in the vector.

Expression of recombinant PA28 $\alpha\beta$ complex using the pET-PA28 $\alpha\beta$ plasmid

Analytical scale: bacteria were transformed with the plasmid pET-PA28 $\alpha\beta$ and grown on solid LB/ampicillin (100 μ g/mL) plates at 37°C overnight. A single colony was selected to grow a 5 mL starter culture overnight in LB/ampicillin (100 μ g/mL) with shaking at 37°C. The starter culture was then inoculated into 30 mL of LB/ampicillin (100 μ g/mL) and incubated at 37°C with shaking until the OD at 600 nm reached 0.8. Expression of recombinant proteins was then induced by addition of IPTG to a final concentration of 0.5 mM. After 3h, bacteria were harvested and collected by centrifugation at 5,000 x g for 15 min. The pellets were re-suspended in 30 mL of PBS and centrifuged again at 3,000 x g for 15 min. The washed pellets were resuspended in 1.5 mL of lysis buffer (PBS, 1% Triton X-100)

and submitted to sonication. Bacterial lysates were clarified at 13,000 x g at 4°C for 15 min to obtain the soluble proteins.

Large scale Expression: The procedures were as for analytical expression, with the following modifications: starter culture, 10 mL; expression performed in 1 L LB/ ampicillin; first wash of the bacteria pellet in 50 mL of PBS; final pellet resuspended in 30 mL of buffer A (20 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA).

High Q 5 mL

The soluble bacterial protein extract was loaded on a High Q 5 mL anion-exchange column equilibrated with buffer A. After collection of the flow-through, the column was washed with 2 column volumes of buffer A, and the bound proteins were eluted with a 100 mL linear NaCl gradient (0-500 mM) in the buffer A. Fractions of 5 mL were collected.

Mono Q 10/10

The active fractions (see below) of the High Q column, eluted at ~100 mM NaCl, were pooled and applied to a 8 mL Mono Q 10/10 column equilibrated with buffer A. After collection of the flow-through, the column was washed with 2 column volumes of buffer A, and the bound proteins were eluted with a 120 mL linear NaCl gradient (0-500 mM) in the buffer A. Fractions of 5 mL were collected.

CHT II 5 mL

The active fractions from the MonoQ 10/10, eluted at ~170 mM NaCl, were pooled and applied to a CHT II 5 mL column equilibrated with buffer PhA (5 mM potassium phosphate, pH 7.5, 1 mM DTT, 50 mM NaCl). After collection of the flow-through, the column was washed with 2 column volumes of buffer PhA, and the bound proteins were eluted with a 50

mL linear gradient of buffer PhB (200 mM potassium phosphate, pH 7.5, 1 mM DTT). Fractions of 5 mL were collected.

Superose 12

The active fractions of the CHT II, eluted at ~25 mM KPh, 45 mM NaCl, were pooled and concentrated using a Vivaspin 15 mL, 10,000 MWCO (Sartorius, Palaiseau, France) to 500 μ L. The concentrated pool was then subjected on a gel filtration using a Superose 12 column equilibrated with buffer A supplemented with 50 mM NaCl. The gel filtration was performed at a flow rate of 0.5 mL/min for 25 mL. Fractions of 500 μ L were collected from 6 mL to 13 mL.

Purification of 20S proteasome

Partially purified 20S proteasome was prepared from HeLa cells, essentially as described for yeast proteasome [31]. Briefly, cytosolic extracts were fractionated by a succession of chromatographic steps including anion exchange, hydroxyapatite and gel filtration using a Superose 6 column. This last step allows resolution of 20S proteasome from 26S proteasome, and the 20S proteasome containing fractions were pooled and conserved at -80°C.

Quantification

The protein concentration was determined with Coomassie Plus™ Protein Assay (Perbio, Brebières, France).

SDS-PAGE analysis

Standard gel

Samples were electrophoresed in Laemmli buffer on a 12% acrylamide gel (containing 12% acrylamide-bisacrylamide (Euromedex, Mundolsheim, France) at a ratio of 37.5:1 and 0.1% SDS at 99.9% purity from Euromedex). Proteins were visualized by staining gels with Brilliant Blue G-colloidal Concentrate (Sigma-Aldrich, Saint Quentin Fallavier, France). PA28 was immunoprobed with anti-PA28 β antibodies, after transfer of the gel to a nitrocellulose membrane (Whatmann).

High-resolution gel

To resolve PA28 α and β subunits, samples were electrophoresed on a 12% high-resolution SDS-PAGE gel (containing 12% acrylamide (Bio-Rad)-bisacrylamide (Bio-Rad, Marnes la coquette, France) at a ratio of 74:1 and 0.1% SDS at 95% purity from Sigma-Aldrich, Saint Quentin Fallavier, France). The gels were stained with coomassie blue. PA28 α and β subunits were immunoprobed with specific antibodies, after transfer of the gel to a nitrocellulose membrane (Whatmann).

In vitro assay for activation of peptidase activity of purified 20S proteasome by PA28

Peptidase activity was assayed using Suc-LLVY-AMC (Bachem, Weil am Rhein, Germany) as a substrate. 5 μ L samples of fractions from each step of PA28 purification were incubated with 1 μ g of 20S proteasome in a 50 μ L assay that contained 20 mM Tris, pH 7.5, 1 mM DTT, 1 mM ATP, 5 mM MgCl₂, 100 μ M Suc-LLVY-AMC. Incubations were carried out at 37°C for 20 min in 96-wells black microplates (Fisher Scientific Bioblock, Illkirch, France). Reactions were stopped with 200 μ L of SDS 1% and fluorescence was monitored with a spectrofluorimeter (BIO-TEK Instrument, Inc., Colmar, France) using filters 360/40 for excitation and 460/40 for emission.

In gel assay for peptidase activity of 20S proteasome

Purified 20S proteasome was incubated without or with purified PA28 $\alpha\beta$ complex at 30°C, for 20 min, in a final volume of 20 μ L, then the mix was loaded on a 4% native PAGE [31]. After electrophoresis, the gel was incubated with 100 μ M Suc-LLVY-AMC in buffer (20 mM Tris, pH 7.5, 1 mM DTT, 1 mM ATP, 5mM MgCl₂) at 37°C, for 20 min. To visualize peptidase activity, the gel was then exposed to UV light and photographed.

Results and discussion

Despite years of *in vitro* studies on PA28 $\alpha\beta$, a rapid and efficient way to produce this heterocomplex was still missing, due to the lack of solubility of PA28 β when expressed alone in bacteria. To overcome this difficulty, we choose to coexpress both PA28 subunits from a single vector in *E. coli*, knowing that insoluble proteins can often be maintained in solution by physical interaction with their(s) partner(s).

Construction of a bicistronic expression plasmid containing both α and β cDNAs

The plasmid pET-PA28 $\alpha\beta$, schematized in figure 1, was constructed by sequential insertion in the pET-17b vector of the two human cDNAs, as described in Materials and Methods. Importantly, a second Ribosome Binding Site (RBS) was inserted before the codon start of the second gene, PA28 β , to allow simultaneous synthesis of both proteins from the single mRNA. The sequence of the plasmid was verified by sequencing and show that the coding sequences of PA28 α and PA28 β are identical to the sequences found in Genbank (NM_176783.1 and NM_002818.2, respectively), except for 2 point mutations in the sequence of PA28 β , a silent A63G mutation and a missense A266C mutation resulting into a Histidine to Proline substitution at position 89 of the protein.

Strain selection for expression of recombinant PA28 $\alpha\beta$

The pET-PA28 $\alpha\beta$ vector was used to transform three different strains of heat-shock competent *E. coli*, BL21 (DE3), BL21 (DE3) codon plus-RP, BL21 (DE3) p-Lys-S (Stratagen). After induction of PA28 subunits expression with IPTG, pellets of 100 μ L samples from uninduced or induced cultures were resuspended in 20 μ L of water and lysed with 10 μ L of 4x Laemmli buffer by incubation at 95°C for 6 min. Samples were electrophoresed on a 12% acrylamide gel. Proteins were visualized by staining the gels with coomassie blue (figure 2A). To analyze whether PA28 β was expressed and soluble, 20 μ L samples of either total extracts or soluble supernatants obtained after centrifugation of the total extracts, were mixed with 10 μ L of 4x Laemmli buffer, resolved by SDS-PAGE and immunoprobed with anti-PA28 β antibodies after transfer on nitrocellulose membranes (figure 2B). For all three strains, the β subunit, which was already significantly expressed before addition of IPTG, remained essentially soluble, showing that coexpression with its PA28 α partner prevented its aggregation, most likely through direct interaction (see below). Further experiments were conducted using the BL21 (DE3) p-Lys-S strain for PA28 expression, as production of soluble PA28 appeared the highest in this context.

High yield expression and purification of recombinant PA28 $\alpha\beta$

After expression of recombinant PA28 subunits in 1 L culture of transformed BL21 (DE3) p-Lys-S, the soluble fraction was prepared and subjected to four successive different chromatographic steps, as outlined in figure 3A and described in Material and Methods. Briefly, after a low resolution anion exchange column (High Q) to discard most unwanted proteins, the PA28 $\alpha\beta$ -containing fractions were subjected to a high resolution anion exchange column (MonoQ), then to hydroxyapatite chromatography and gel filtration. At each step of purification, the fractions containing the PA28 α and β subunits were identified by coomassie blue-staining or western-blot of the gels, and by their ability to activate peptidase activity of

purified 20S proteasome, using suc-LLVY-AMC as a substrate. Parallel assays without addition of 20S proteasome were performed to exclude possible effects of a contaminating bacterial protease. Figures 3B and 3C show the analysis of the last step of the purification (gel filtration on Superose 12) of a typical experiment. The major peak of proteins (DO at 280 nm, blue line) tightly correlated with the peak of activation of 20S proteasome peptidase activity (dotted red line), whereas the corresponding fractions had no peptidase activity by themselves (not shown). These fractions (~200 kDa) were analyzed by 12% high-resolution SDS-PAGE in order to resolve both PA28 subunits, and stained with coomassie blue (figure 3C). They contain two ~28 kDa polypeptides, that correspond to PA28 α and β subunits, as shown in figure 4. Of note, the two subunits are coeluted in an ~200 kDa complex that corresponds to the expected size for PA28 [9, 11, 13, 32]. The PA28 $\alpha\beta$ -containing fractions were pooled, and kept in aliquots at -20°C. Under these conditions, the complex remains active, as shown by its ability to stimulate 20S proteasome peptidase activity when assayed either in tube (not shown) or by the "gel overlay" method (figure 5).

In a typical experiment, the recovery of pure PA28 $\alpha\beta$ from 1 L bacterial culture (~250 mg total soluble proteins) was approximately 1.5 to 2 mg.

The results detailed in this article demonstrate the usefulness of a bicistronic vector to coexpress PA28 α and β subunits. Using this strategy, the heterocomplex forms in the *E. coli* host, probably in a cotranslational process. Once assembled, the active complex remains stable and soluble, and can be readily purified by classical chromatography. A possibility would have been to tag one of the subunit at its N-terminal end, in order to allow affinity purification of the complex, but we preferred a strategy yielding an unmodified recombinant complex.

When different purified samples were analyzed by SDS-PAGE, we noticed some variation in the apparent stoichiometry of the subunits in the complex. Although the slowest migrating

band, corresponding to PA28 β , was less strongly stained than the fastest migrating one (PA28 α), the apparent ratio of the two bands varied slightly from sample to sample. Although we did not precisely measure this variation, it is noteworthy that during the chromatography on hydroxyapatite, sometimes the active fractions were not eluted in one discrete peak. When this occurred, although gel filtration analysis of the different peaks always yielded complexes of identical sizes, the apparent ratio of the two subunits showed minor but detectable variations. These observations suggest that, even though the two subunits are expected to be produced at similar levels, their assembly might not be a tightly controlled process and might lead to formation of heterogeneous complexes, at least in *E. coli*. Whether this reflects a similar heterogeneity of PA28 complexes in eukaryotic cells remains to be investigated. In any case, since the exact stoichiometry of the native mammalian PA28 is still a matter of debate [12, 13, 15], and since despite the possible variation of stoichiometry of the recombinant complex we always obtained an active heterocomplex of a single apparent molecular weight of ~200 kDa (compatible, as the endogenous mammalian PA28, with an heterohexamer or an heteroheptamer), we believe that the recombinant PA28 complex produced by the procedure described in this article represents a very good alternative to the endogenous PA28 in order to study the structural and biochemical properties of this complex *in vitro*.

Abbreviations used:

IPTG, Isopropyl- β -D-thiogalactopyranoside; DTT, 1,4-dithiothreitol; Suc-LLVY-AMC, Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; IgG, immunoglobulin G; SDS, Sodium dodecyl sulfate; TEMED, N,N,N',N'-Tetramethylethylenediamine.

References

- [1] A. Ciechanover, The ubiquitin-proteasome pathway: on protein death and cell life, *EMBO J*, 17 (1998) 7151-7160
- [2] M.H. Glickman, A. Ciechanover, The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction, *Physiol Rev*, 82 (2002) 373-428
- [3] O. Coux, K. Tanaka, A.L. Goldberg, Structure and functions of the 20S and 26S proteasomes, *Annu Rev Biochem*, 65 (1996) 801-47
- [4] D. Voges, P. Zwickl, W. Baumeister, The 26S Proteasome: A Molecular Machine Designed for Controlled Proteolysis, *Annu. Rev. Biochem.*, 68 (1999) 1015-1068
- [5] M. Groll, L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H.D. Bartunik, R. Huber, Structure Of 20S Proteasome From Yeast At 2.4 Å Resolution, *Nature*, 386 (1997) 463-471
- [6] M. Unno, T. Mizushima, Y. Morimoto, Y. Tomisugi, K. Tanaka, N. Yasuoka, T. Tsukihara, The Structure of the Mammalian 20S Proteasome at 2.75 Å Resolution, *Structure (Camb)*, 10 (2002) 609-18.
- [7] F.G. Whitby, E.I. Masters, L. Kramer, J.R. Knowlton, Y. Yao, C.C. Wang, C.P. Hill, Structural basis for the activation of 20S proteasomes by 11S regulators, *Nature*, 408 (2000) 115-20
- [8] A. Forster, C.P. Hill, Proteasome degradation: enter the substrate, *Trends Cell Biol*, 13 (2003) 550-3
- [9] C.P. Ma, C.A. Slaughter, G.N. DeMartino, Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain), *J Biol Chem*, 267 (1992) 10515-23
- [10] M. Rechsteiner, C. Realini, V. Ustrell, The proteasome activator 11 S REG (PA28) and Class I antigen presentation, *Biochem J*, 345 (2000) 1-15

- [11] K. Ahn, M. Erlander, D. Leturcq, P.A. Peterson, K. Fruh, Y. Yang, In Vivo Characterization Of the Proteasome Regulator Pa28, *J Biol Chem*, 271 (1996) 18237-18242
- [12] X. Song, J.D. Mott, J. von Kampen, B. Pramanik, K. Tanaka, C.A. Slaughter, G.N. DeMartino, A model for the quaternary structure of the proteasome activator PA28, *J Biol Chem*, 271 (1996) 26410-7
- [13] Z. Zhang, A. Krutchinsky, S. Endicott, C. Realini, M. Rechsteiner, K.G. Standing, Proteasome activator 11S REG or PA28: recombinant REGalpha/REGbeta hetero-oligomers are heptamers¹, *Biochemistry*, 38 (1999) 5651-8
- [14] J.R. Knowlton, S.C. Johnston, F.G. Whitby, C. Realini, Z. Zhang, M. Rechsteiner, C.P. Hill, Structure of the proteasome activator REGalpha (PA28alpha), *Nature*, 390 (1997) 639-43
- [15] J. Li, M. Rechsteiner, Molecular dissection of the 11S REG (PA28) proteasome activators, *Biochimie*, 83 (2001) 373-83.
- [16] C.W. Gray, C.A. Slaughter, G.N. Demartino, PA28 activator protein forms regulatory caps on proteasome stacked rings, *J Mol Biol*, 236 (1994) 7-15
- [17] K.B. Hendil, S. Khan, K. Tanaka, Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes, *Biochem J*, 332 (1998) 749-54
- [18] N. Tanahashi, Y. Murakami, Y. Minami, N. Shimbara, K.B. Hendil, K. Tanaka, Hybrid proteasomes. Induction by Interferon-gamma and contribution to ATP-dependent proteolysis, *J Biol Chem*, 275 (2000) 14336-45
- [19] C. Realini, C.C. Jensen, Z. Zhang, S.C. Johnston, J.R. Knowlton, C.P. Hill, M. Rechsteiner, Characterization of recombinant REGalpha, REGbeta, and REGgamma proteasome activators, *J Biol Chem*, 272 (1997) 25483-92

- [20] M. Groettrup, A. Soza, M. Eggers, L. Kuehn, T.P. Dick, H. Schild, H.G. Rammensee, U.H. Koszinowski, P.M. Kloetzel, A Role For the Proteasome Regulator PA28-Alpha In Antigen Presentation, *Nature*, 381 (1996) 166-168
- [21] A.F. Kisselev, T.N. Akopian, K.M. Woo, A.L. Goldberg, The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation, *J Biol Chem*, 274 (1999) 3363-71
- [22] T. Preckel, W.P. Fung-Leung, Z. Cai, A. Vitiello, L. Salter-Cid, O. Winqvist, T.G. Wolfe, M. Von Herrath, A. Angulo, P. Ghazal, J.D. Lee, A.M. Fourie, Y. Wu, J. Pang, K. Ngo, P.A. Peterson, K. Fruh, Y. Yang, Impaired immunoproteasome assembly and immune responses in PA28(-/-) mice, *Science*, 286 (1999) 2162-5
- [23] S. Murata, H. Udono, N. Tanahashi, N. Hamada, K. Watanabe, K. Adachi, T. Yamano, K. Yui, N. Kobayashi, M. Kasahara, K. Tanaka, T. Chiba, Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta, *EMBO J*, 20 (2001) 5898-5907.
- [24] P. Cascio, M. Call, B.M. Petre, T. Walz, A.L. Goldberg, Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes, *EMBO J*, 21 (2002) 2636-45.
- [25] G.A. Ordway, P.D. Neuffer, E.R. Chin, G.N. DeMartino, Chronic contractile activity upregulates the proteasome system in rabbit skeletal muscle, *J Appl Physiol*, 88 (2000) 1134-1141
- [26] G. Fuertes, J.J. De Llano, A. Villarroya, A.J. Rivett, E. Knecht, Changes in the proteolytic activities of proteasomes and lysosomes in human fibroblasts produced by serum withdrawal, amino acid deprivation and confluent conditions, *Biochem J*, Pt (2003)
- [27] P. Gromov, G.L. Skovgaard, H. Palsdottir, I. Gromova, M. Ostergaard, J.E. Celis, Protein profiling of the human epidermis from the elderly reveals up-regulation of a signature of interferon-gamma-induced polypeptides that includes manganese-superoxide dismutase

and the p85beta subunit of phosphatidylinositol 3-kinase, *Mol Cell Proteomics*, 2 (2003) 70-84

[28] Y. Minami, H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka, I. Yahara, A Critical Role for the Proteasome Activator PA28 in the Hsp90-dependent Protein Refolding, *J Biol Chem*, 275 (2000) 9055-9061

[29] M. Minami, F. Shinozaki, M. Suzuki, K. Yoshimatsu, Y. Ichikawa, Y. Minami, The proteasome activator PA28 functions in collaboration with Hsp90 in vivo, *Biochem Biophys Res Commun*, 344 (2006) 1315-1319

[30] X. Song, J. von Kampen, C.A. Slaughter, G.N. DeMartino, Relative functions of the alpha and beta subunits of the proteasome activator, PA28, *J Biol Chem*, 272 (1997) 27994-8000

[31] M. Glickman, O. Coux, Purification and characterization of proteasomes from *Saccharomyces cerevisiae*, *Curr Protoc Protein Sci*, Chapter 21 (2001) Unit 21 5

[32] L. Kuehn, B. Dahlmann, Proteasome Activator Pa28 and Its Interaction With 20 S Proteasomes, *Arch Biochem Biophys*, 329 (1996) 87-96

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Figure Legends

Figure 1 : Diagram of the pET-PA28 $\alpha\beta$ bicistronic plasmid constructed for this study:

The plasmid was derived from the pET-17b vector (Novagen), in which both PA28 α and β cDNAs, each preceded by a Ribosome Binding Site (RBS) were inserted under the control of a T7 promoter. Amp R, ampicillin resistance marker; T7 prom, T7 promoter; T7 term, T7 terminator. The cloning sites, Xho I, EcoR V, BamH I and Nde I, are shown in black and the restriction sites, BamH I and Nde I, present in the PA28 β sequence are shown in grey.

Figure 2 : Expression of recombinant PA28 $\alpha\beta$ in various *E. coli* strains. A-

Coomassie blue-stained 12% SDS-PAGE gel of protein extracts from *E. coli* strains transformed with the plasmid pET-PA28 $\alpha\beta$, and treated (+) or not (-) with 0.5 mM IPTG. (1) BL21(DE3), (2) BL21(DE3) codon plus-RP, (3) BL21(DE3) p-Lys-S. Molecular weight markers (M) are Prestained Protein Marker, Broad Range (6-175 kDa) from Biolabs. Arrow indicates the presence of PA28 $\alpha\beta$ bands at the expected (~28 kDa) position. B- Western blot analysis of total and soluble proteins of the extracts shown in A, using anti-PA28 β antibodies. After lysis of the bacteria, insoluble proteins were removed by centrifugation at 13,000 g for 15 min. Proteins were resolved on 12% SDS-PAGE gel, transferred on a nitrocellulose membrane (Whatmann) and blotted with anti-PA28 β antibodies. T, total extract before centrifugation. S, supernatant after centrifugation.

Figure 3 : A. Purification scheme of recombinant PA28 $\alpha\beta$ complex. Details of the

purification procedure are described under Materials and methods. B. Final step of purification: gel filtration on superose 12. Details of the chromatography are described under Materials and methods. Fractions 5 to 24 were incubated with purified 20S proteasomes for 20 min. at 30°C, then assayed on peptidase activity, using suc-LLVY-AMC as a substrate

(red dotted line). Blue line: optical density at 280nm. The black arrowheads indicate the position of MW markers: from left to right, Ferritin (440 kDa), Aldolase (158 kDa), Ovalbumin (43 kDa), Chymotrypsinogen (25 kDa). **C. SDS-PAGE analysis of the peak fractions.** Coomassie blue-stained 12% high resolution SDS-PAGE gel of 10 μ L samples of fractions 12, 13 and 14.

Figure 4 : SDS-PAGE and western blot analyses of purified PA28 $\alpha\beta$. **A-** Coomassie blue-stained 12% high resolution SDS-PAGE gel of purified PA28 $\alpha\beta$ complex. **B and C-** Western blot detection of PA28 subunits, using anti-PA28 α and anti-PA28 β antibodies, respectively.

Figure 5 : Activation of 20S proteasome by purified recombinant PA28 $\alpha\beta$ complex. 6.3 μ g of partially purified 20S proteasome was incubated for 20 min. at 30°C, without (-) or with (+) 1.2 μ g of purified recombinant PA28 $\alpha\beta$, and assayed by peptide overlay after electrophoresis on 4% polyacrylamide gel in native conditions. Degradation of the peptide Suc-LLVY-AMC by the proteasome results into a fluorescent signal due to the free AMC and thus roughly proportional to the activity of the complex.

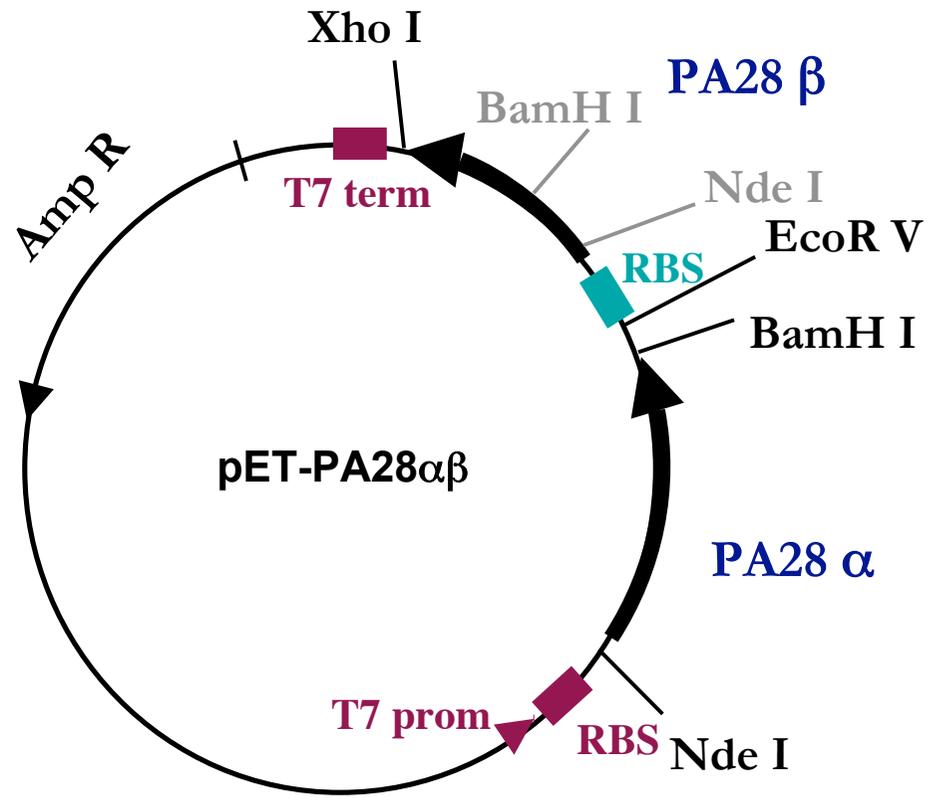


Figure 1

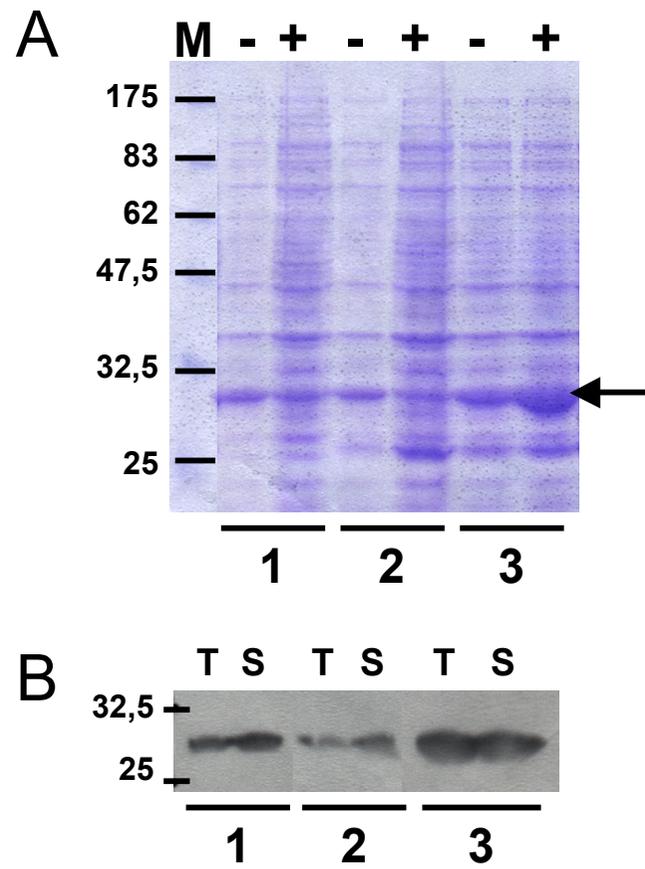


Figure 2

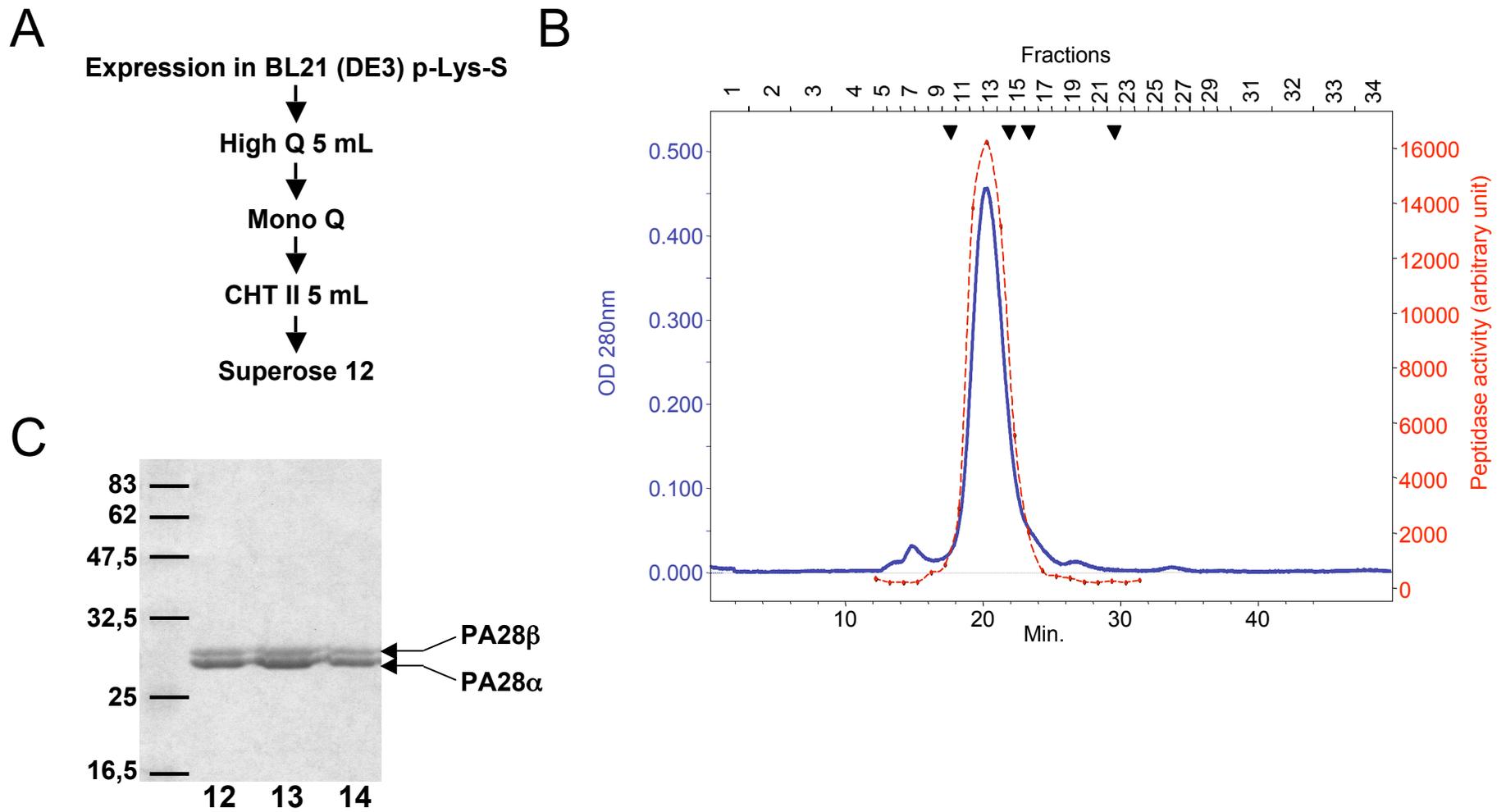


Figure 3

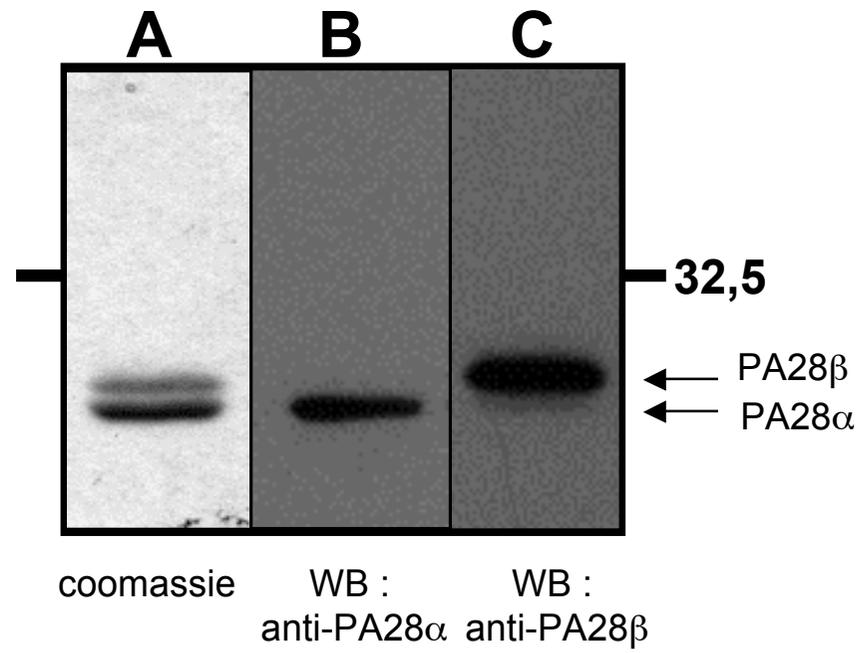


Figure 4

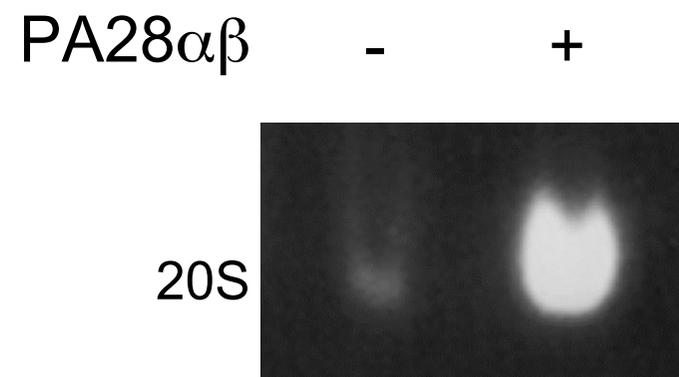


Figure 5