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The proteasome maturation protein POMP facilitates major steps of 20S proteasome formation at the endoplasmic reticulum

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The quality control of proteins mediated by the plasticity of the proteasome system is regulated by the timely and flexible formation of this multisubunit proteolytic enzyme complex. Adaptable biogenesis of the 20S proteasome core complex is therefore of vital importance for adjusting to changing proteolytic requirements. However, the molecular mechanism and the cellular sites of mammalian proteasome formation are still unresolved. By using precursor complex-specific antibodies, we now show that the main steps in 20S core complex formation take place at the endoplasmic reticulum (ER). Thereby, the proteasome maturation protein (POMP)—an essential factor of mammalian proteasome biogenesis—interacts with ER membranes, binds to α_{1-7} rings, recruits β-subunits stepwise and mediates the association of mammalian precursor complexes with the ER. Thus, POMP facilitates the main steps in 20S core complex formation at the ER to coordinate the assembly process and to provide cells with freshly formed proteasomes at their site of function.

Keywords: proteasome; assembly; POMP; localization; ER
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INTRODUCTION

Proteolysis regulated by the proteasome has a central role in the control and maintenance of basic cellular processes as well as immune surveillance (Kloetzel, 2001). Its three active sites reside in the subunits β1, β2 and β5 of the 20S core complex (Groll *et al*, 1997), and can be exchanged by β1i/LMP2, β2i/MECL1 and β5i/LMP7 on cytokine stimulation for immune adaptation of the proteasome (Kloetzel, 2001).

Assembly of 17 different mammalian subunits into a defined $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ heteroheptameric ring structure of either constitutive (c20S) or immuno-(i20S) proteasomes requires a multistep biogenesis programme through distinct assembly intermediates (pre20S) and the assistance of helper proteins. For mammals, three different pre20S have been described so far, α_{1-7} rings (Hirano *et al*, 2005), 13S or the 16S complexes (Frentzel *et al*, 1994; Nandi *et al*, 1997; Schmidtke *et al*, 1997; Heink *et al*, 2005). α_{1-7} Ring assembly is facilitated by multiple proteasome assembly chaperone (PAC) proteins (Hirano *et al*, 2005, 2006), whereas the arrangement of β-subunits on α_{1-7} rings remains elusive. We previously showed that proteasome maturation protein (POMP) is exclusively contained in pre20S and that POMP is essential for proβ5 subunit recruitment and cell viability (Witt *et al*, 2000; Heink *et al*, 2005). During the final steps of 20S formation, β1, β2 and β5 are activated by autocatalytic removal of propeptides (Frentzel *et al*, 1994; Nandi *et al*, 1997) and POMP becomes degraded (Ramos *et al*, 1998; Heink *et al*, 2005).

Depending on their physiological state, pre20S can represent up to 30% of the immunoreactive proteasome particles in mammalian cells (Nandi *et al*, 1997). Antibodies directed against any of the α- or β-subunits cannot differentiate between mature or nascent 20S complexes, so until now sites of proteasome function could not be distinguished from sites of proteasome formation. To overcome this problem, we used two pre20S-specific antibodies to localize pre20S complexes at the endoplasmic reticulum (ER) and identified POMP as a mediator for coordinated completion of proteasome biogenesis at the ER.

RESULTS AND DISCUSSION

Proteasome precursors are localized at the ER

To study the intracellular localization of pre20S, we used two pre20S-specific antibodies, AbC8 and AbPOMP. AbC8 recognizes a pre20S-specific conformation of the α7-subunit (Nandi *et al*, 1997) and consistently precipitated 13S complexes (α_{1-7}, proβ2, proβ6, β3, β4, POMP; Fig 1A,B). AbPOMP has been shown to detect pre20S exclusively (Witt *et al*, 2000; Heink *et al*, 2005). By contrast, antibodies directed against c20S mainly precipitated mature 20S proteasomes (Fig 1A).

Both AbC8 and AbPOMP showed a predominant localization of pre20S complexes at the ER in confocal images. Co-staining of AbC8 with the ER marker calnexin, an integral ER membrane protein, or ERAP1, a luminal ER aminopeptidase (Saveanu *et al*,

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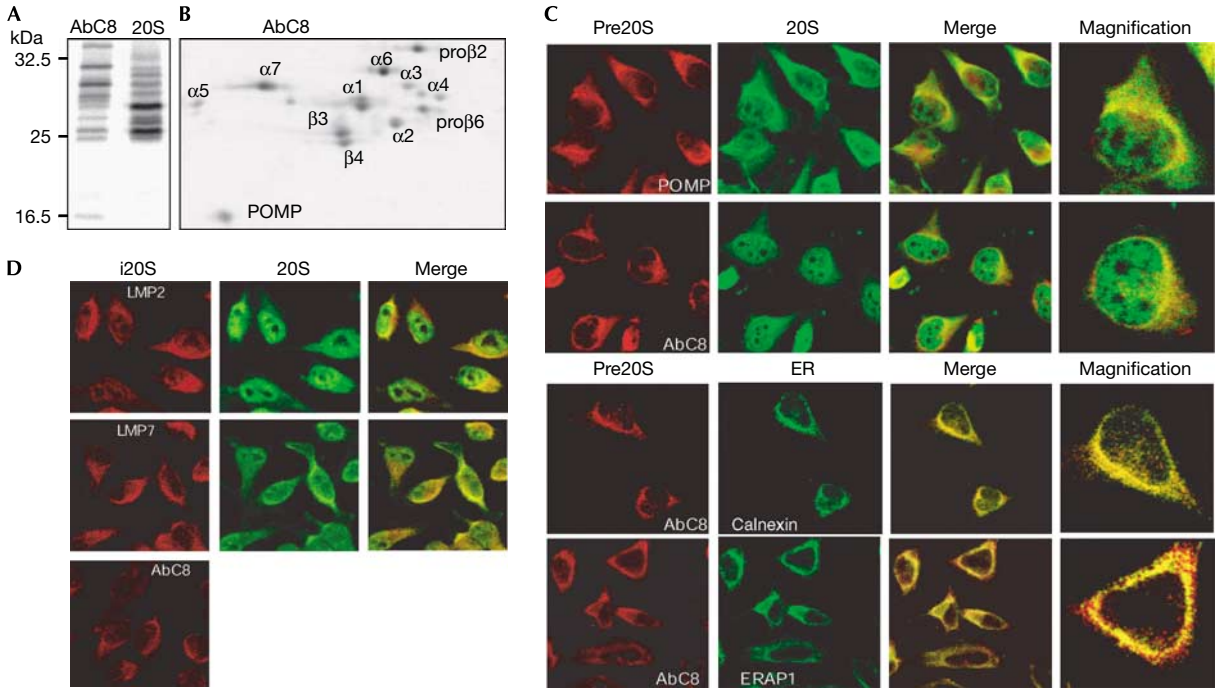

Fig 1 | Pre20S subunits were detected at the endoplasmic reticulum. (**A**) AbC8 recognized pre20S and the 20S antibody mature 20S as shown by autoradiographic protein patterns precipitated from metabolically pulse-labelled HeLa cells. (**B**) Two-dimensional separation of AbC8 precipitates showing 13S complexes (α_{1-7}, proβ2, proβ6, β3, β4 and POMP). (**C**) Confocal image of pre20S distribution (AbC8, POMP) with co-staining for 20S, calnexin or ERAP1 in HeLa cells. Colocalization is shown in the merge panels. (**D**) Confocal image of i20S distribution (LMP2/β1i and LMP7/β5i) with co-staining for 20S HeLa cells stimulated with interferon-γ (100 U/ml, 24 h). i20S precursors (prei20S) stained with pAbC8 gave a similar distribution pattern as i20S. Ab, antibody; ER, endoplasmic reticulum; ERAP, ER aminopeptidase; POMP, proteasome maturation protein.

2005), almost completely matched with the staining of pre20S (Fig 1C). This typical ER-restricted pre20S staining pattern was also observed in other mammalian cells (supplementary Fig 1 online) and was shown in published pictures of POMP localization, but with a different interpretation (Hoefer *et al*, 2006). We did not find colocalization of pre20S with nuclear pores or with vimentin (data not shown). This contrasts with observations made in yeast, in which most of the pre20S complexes were detected inside the nucleus (Lehmann *et al*, 2002), and highlights data that proteasome formation in mammals differs from that in yeast (Witt *et al*, 2000; Heink *et al*, 2005; Hirano *et al*, 2005). c20S were found in the cytoplasm, at the ER and in the nucleus (Fig 1C), reflecting their sites of function (Wojcik & DeMartino, 2003).

Localization of i20S using AbLMP2 (β1i) and AbLMP7 (β5i) was predominantly confined to the ER (Fig 1D), as reported previously (Wojcik & DeMartino, 2003). As AbC8—which also precipitates i20S precursors (Nandi *et al*, 1997; Heink *et al*, 2005)—gave a fluorescence image almost identical to that obtained for AbLMP2 or AbLMP7 (Fig 1D), we concluded that i20S precursors were also localized at the ER (Fig 1D). This corresponds with the main site of i20S function in major histocompatibility complex (MHC) class I antigen presentation (Kloetzel, 2001).

Most of the 13S complexes reside ER-bound in the cell

Supporting the cytological data, proβ5 and POMP as markers for pre20S were indeed found mainly in membrane-enriched subcellular fractions, whereas mature β5 was detected in all fractions examined (Fig 2A). As further proof, we carried out membrane flotation experiments and defined ER-containing fractions by the presence of calnexin (Fig 2B). These immunoblot analyses, which determine a steady-state situation of subcellular proteasome distribution, showed a considerable but not complete flotation of all β-subunit proforms with membranes. POMP floated almost completely with ER membranes, thus verifying that pre20S subunits are localized at the ER. Fully processed β-subunits reflecting mature proteasomes were also detected at the ER, which is in agreement with proteasome function in ER protein quality control (Meusser *et al*, 2005).

To investigate whether the ER-bound pre20S subunits are organized as complexes and to minimize β-subunit processing during the preparation, pulse-labelled pre20S or 20S complexes were precipitated from cytoplasmic or floated membrane fractions. Both the cytoplasmic and the membrane fractions contained pre20S complexes as well as 20S complexes (Fig 2C). However, quantitative evaluation showed 75% of the precipitated pre20S material to be associated with the ER, which was even more pronounced (88%) when the amount of ER-associated POMP was calculated (Fig 2D). As most pre20S material was precipitated from microsomal fractions, we reasoned that POMP-containing pre20S complexes predominantly reside in an ER-bound state. Thus, the ER might represent the predominant site of mammalian proteasome formation.

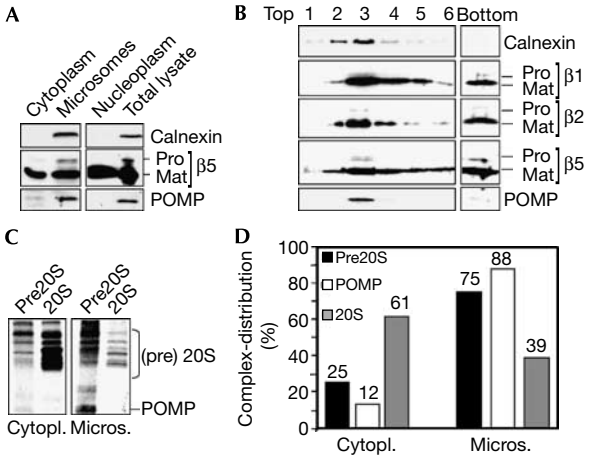

Fig 2 | The majority of pre20S resides endoplasmic reticulum-bound in the cell. (**A**) Subcellular fractions of HeLa cells analysed for β5 (proβ5, matβ5), POMP or calnexin (ER marker) in immunoblots. (**B**) Active site β-subunit proforms and POMP floated with isolated microsomes and calnexin. Gradient fractions were analysed for β1 (proβ1, matβ1), β2 (proβ2, matβ2), β5 (proβ5, matβ5), POMP or calnexin (ER marker) in immunoblots. (**C**) Most pre20S complexes were bound to microsomes. Autoradiogram of pre20S (AbC8) or 20S complexes from cytoplasmic (Cytopl.) or floated microsomal (Micros.) fractions of pulse-labelled HeLa cells is shown. The typical pattern of pre20S, POMP or 20S is indicated. A representative experiment is shown. (**D**) Quantitative evaluation of the experiment in (**C**) by phosphoimaging. Numbers are distribution of pre20S, POMP and 20S proteasomes. ER, endoplasmic reticulum; Mat, matured form; POMP, proteasome maturation protein; Pro, proform.

POMP interacts directly with membranes

Considering the number of different subunits and hence the complexity of proteasome assembly, we assumed that POMP could directly bind to the ER. When expressed with canine pancreatic microsomal membranes (CMMs), *in vitro*, POMP was recovered from the pelleted membrane fraction (Fig 3A). However, in the absence of membranes, POMP was completely retained in the soluble fraction, excluding the possibility that the observed membrane interaction was due to the formation of pelletable POMP aggregates. Luciferase, which acted as a negative control, remained completely in the soluble fraction (Fig 3A). Interestingly, membrane–POMP interactions did not require any additional proteins *in vitro*, as POMP also bound to artificial liposomes. POMP was solubilized by agents that strip peripheral membrane proteins (pH 11, urea), so it seems likely that POMP is a peripheral membrane protein (Fig 3B). To test further whether the binding of POMP to membranes occurred in a stoichiometric manner, POMP or luciferase was expressed in excess in the presence of increasing amounts of CMMs. Densitometric quantification showed a direct correlation between the amount of added microsomes and the amount of precipitated POMP. Luciferase did not interact with CMMs (Fig 3C).

Next, we investigated the stability of POMP association with ER membranes using floated microsomes. POMP remained stably attached to floated microsomes even at a salt concentration of 0.75 M KCl (Fig 3D), whereas other proteasomal subunits were stripped from microsomes at lower salt concentrations (data not shown), confirming the *in vitro* data. In summary, these experiments strongly imply a significant and stable interaction of POMP with microsomal membranes *in vivo*, and indicate that full-length POMP shows features of a peripheral membrane protein.

Different pre20S complexes exist in an ER-bound state

To gain more insight into the direct interaction partners of POMP, we observed by using protein-interaction analyses that POMP can bind to all proβ-subunits, including the immunosubunits (supplementary Fig 2A online). This extends the recruiting function of POMP shown experimentally for both β5-subunits (Heink *et al*, 2005) to all other β-type subunits. In addition, POMP also interacted with the α-subunits α3, α4 and α7 (supplementary Fig 2A online). In particular, the α7-subunit was previously assumed to have a pivotal role in α-ring assembly (Gerards *et al*, 1997; Apcher *et al*, 2004; Hirano *et al*, 2005). In support of this, we found POMP to sediment in gradient fractions 5–7 containing α-rings and Flag-tagged PAC2 (supplementary Fig 2E online) and to migrate in the same complexes in native gels (Fig 4B). Moreover, POMP can be precipitated with α4 from these fractions using either an α6 antibody or a Flag antibody (Fig 4A).

To assess whether POMP binds to α-rings, we established an *in vitro* system for α-ring formation and to circumvent the problem of continuously proceeding assembly of pre20S isolated from cells. The complexes formed were separated by sucrose gradient ultracentrifugation (supplementary Fig 2B,C online). Native gel analysis of fractions 5–7 showed a single band corresponding to the theoretical size of an α-ring (supplementary Fig 2D online). Immunoprecipitates from fractions 5–7 with an α6-antibody showed a complex of α_{1-7} and POMP, indicating that POMP interacts with α-rings (Fig 4C). In agreement with a recent report, PACs were dispensable for α-ring assembly *in vitro* (Hirano *et al*, 2005). POMP–α-ring complexes formed *in vitro* were soluble in the absence of microsomes and pelleted together with membranes, whereas α-subunits expressed alone had only a weak tendency to pellet (Fig 4D). Thus, we concluded that POMP–α-rings exist in an ER-bound state. Further support for our hypothesis comes from studies that localize PAC1 (also Down syndrome critical region gene 2 (DSCR2)) to the cytoplasm as well as to the ER (Possik *et al*, 2004; Vesa *et al*, 2005).

To study the nature of ER-bound pre20S, 13S complexes were immunoprecipitated from floated membranes that showed the same composition as that from total lysates (Figs 1A,4D). By using an α6-antibody for immunoprecipitation from the same material, we also detected 16S complexes (α_{1-7}, β3, β4, proβ1, proβ2, proβ5, proβ6 and proβ7; Fig 4E). In fact, these experiments show that β-subunits are incorporated stepwise into the nascent complex at the ER. The presence of small amounts of mature β2, β6 and β7 indicates ongoing maturation and shows that short-lived 13S and 16S complexes, which can also be precipitated with other antibodies, are on-pathway intermediates. We were unable to detect α-rings with this approach, most likely due to the low abundance and short half-life of these complexes (Hirano *et al*, 2005, 2006). In summary, we concluded that the main steps of 20S proteasome assembly occur at the ER.

POMP mediates the association of pre20S with the ER

The essential function of POMP in proteasome assembly (Heink *et al*, 2005), together with its almost exclusive localization at the

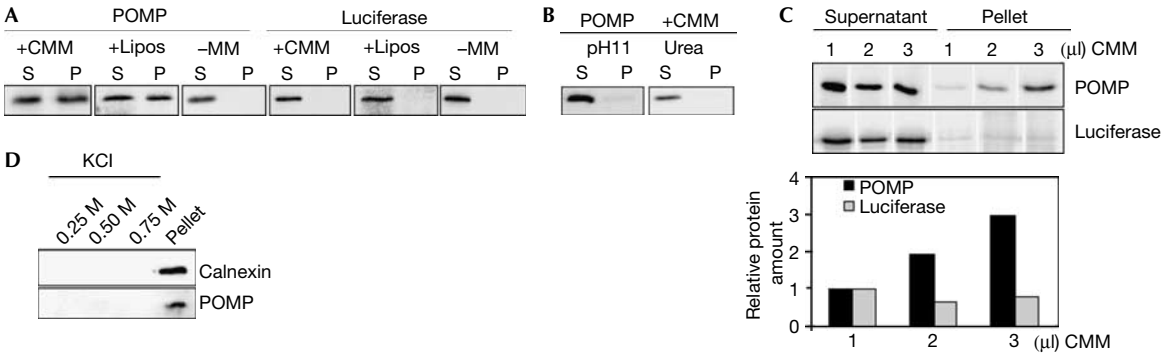

Fig 3 | POMP can interact directly with membranes. (**A**) POMP and firefly luciferase were expressed *in vitro* and radiolabelled in reticulocyte lysate in the presence of CMM (+CMM) or liposomes (+Lipos) or in the absence of membranes (−MM). The membranes were separated by centrifugation, and membrane-bound (pellet, P) or unbound (soluble, S) material was analysed by autoradiography. (**B**) POMP was removed from CMMs by washing the membranes at pH11 or with urea. (**C**) Binding of POMP to membranes occurred in a stoichiometric manner. POMP and luciferase were expressed in the presence of increasing amounts of CMMs. Radioactive material from the pellet and the soluble fraction was evaluated by phosphoimaging. A graph of the quantitative evaluation of pelleted material (POMP versus luciferase) is shown. The pixel density of the 1 µl sample was set to 1. A representative experiment is shown. (**D**) The association of POMP with floated membranes is salt stable. Supernatants of each wash step were immunoblotted for POMP or calnexin. CMM, canine pancreatic microsomal membrane; POMP, proteasome maturation protein.

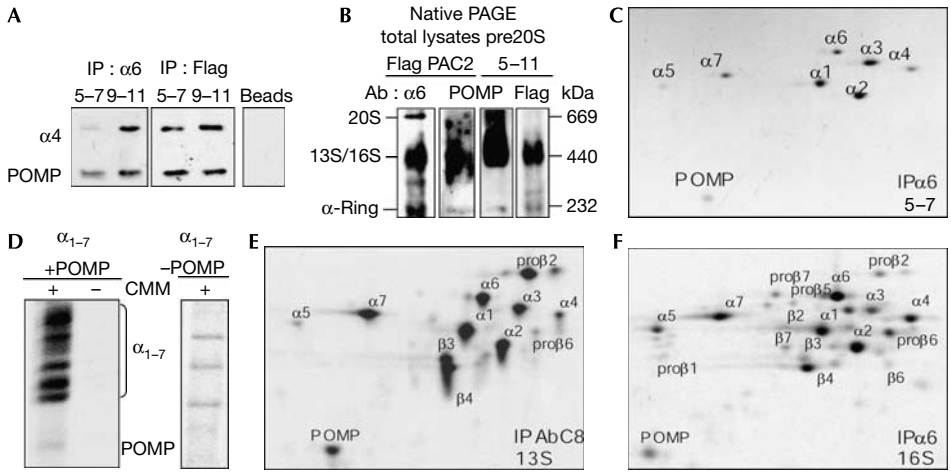

Fig 4 | Different pre20S complexes exist in an endoplasmic reticulum-bound state. (**A**) POMP can be precipitated with α4 from fractions 5–7 (α-rings) and 9–11 (13S) of HeLa cells stably expressing Flag–PAC2 using an α6-Ab or Flag-Ab. Control: beads without fractions. (**B**) Native PAGE analysis of POMP and PAC-containing complexes from HeLa lysates stably expressing Flag–PAC2 or enriched pre20S fractions (5–11) immunodetected for α6, POMP and Flag–PAC2. 20S or pre20S complexes (α-rings, 13S/16S) are indicated. (**C**) Two-dimensional separation of immunoprecipitated (α6-Ab) fractions 5–7 of *in vitro*-formed α-ring–POMP complexes. (**D**) POMP-containing α-rings interact with membranes. Pellet fractions of α-ring complexes formed *in vitro* in the presence (+) or absence (−) of CMMs and POMP are shown. (**E**) Two-dimensional separation of immunoprecipitated (AbC8) 13S complexes (α_{1-7}, proβ2, proβ6, β3, β4 and POMP) from membrane floated material of pulse-labelled cells. (**F**) Two-dimensional separation of immunoprecipitated (α6-Ab) 16S complexes (α_{1-7}, proβ1, proβ2, proβ6, β3, β4, proβ5, proβ7 and POMP) from membrane floated material of pulse-labelled cells. Note the low methionine content of α4 and the abnormal migration behaviour of α5 due to the low isoelectric point. Ab, antibody; CMM, canine pancreatic microsomal membrane; ER, endoplasmic reticulum; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; POMP, proteasome maturation protein.

ER, raised the possibility that POMP might be involved in the binding of distinct pre20S to the ER membrane. In this case, elimination of POMP ought to result in depletion of pre20S complexes at the ER. Being aware that downregulation of POMP can induce apoptosis (Heink *et al*, 2005), we consequently silenced POMP expression for time periods that preserved the viability of the cells. Indeed, depletion of POMP abolished the association of β-subunit proforms with the ER, whereas calnexin and the peripheral ER membrane protein p97 were not affected (Fig 5A). In control cells, most of the POMP, as well as proβ1 and proβ5, sedimented together with calnexin in the membrane fractions, whereas mature β5 was detected in both fractions in

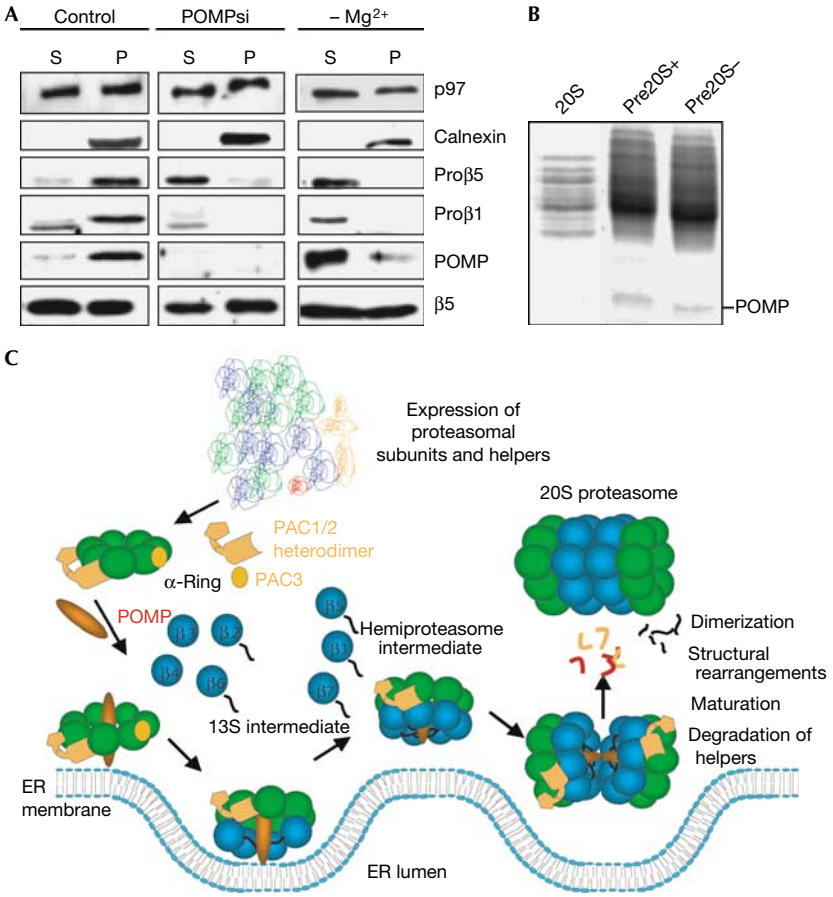

Fig 5 | POMP mediates the binding of pre20S to the endoplasmic reticulum. (**A**) Depletion of POMP or magnesium ($-Mg^{2+}$) abolishes the interaction of pre20S components with the ER membrane. Soluble (S) and membrane-bound (pellet, P) subcellular fractions were separated by centrifugation and analysed in immunoblots for pre20S (proβ1, proβ5 and POMP) or calnexin in mock-transfected (control) and POMP-depleted (POMPsi) cells. Magnesium depletion showed the same result. (**B**) Magnesium depletion did not affect the integrity of the precursor complex. A coomassie-stained gel of immunoprecipitations of pre20S with AbC8 from the membrane fraction in the presence (pre20S+) or absence (pre20S−) of magnesium in comparison to 20S proteasomes is shown. (**C**) A model of 20S proteasome formation. After the expression of all subunits and helpers, α-ring assembly is facilitated by the PAC proteins in the cytoplasm and targeted to the ER. POMP mediates ER binding, recruits the remaining β-subunits into the nascent complex and supports final proteasome maturation. Thereby, POMP is degraded by the newly formed 20S proteasome. Ab, antibody; ER, endoplasmic reticulum; POMP, proteasome maturation protein.

similar amounts (Fig 5A). In agreement with the above hypothesis, these experiments show that mammalian POMP mediates the association of pre20S with the ER. As POMP depletion might affect complex integrity, we also tested for other conditions to remove pre20S from the ER. Interestingly, the binding of pre20S to the membrane, but not their complex integrity (Fig 5B), requires magnesium (Fig 5A). However, the binding of 20S proteasomes is magnesium- and POMP-independent, as shown by the presence of mature β5-subunit (Fig 5A).

In conclusion, our experiments underscore the vital importance of POMP in mammalian proteasome formation. We have previously shown that POMP has an essential recruiting function for incorporation of both β5-subunits into the 20S complex (Heink *et al*, 2005). Thus, interaction of POMP with all β-subunits together with POMP–α-ring interaction provides a plausible explanation for the mechanism of how β-subunits are attached to α-rings. This extends recent findings on α-ring assembly by PACs (Hirano *et al*, 2005, 2006). Together with the almost exclusive localization of pre20S complexes at the ER, our data show that essential steps take place at the ER by a POMP-mediated mechanism. However, we cannot completely exclude the possibility that there are minor proteasome assembly pathways in the cytoplasm or the nucleus.

On the basis of the experimental data provided here and the previously published results, we are now able to propose a revised model for mammalian proteasome assembly: α-ring assembly is facilitated by PACs and targeted to the ER either by PAC1 or by interaction with POMP. There, POMP recruits the remaining β-subunits into the nascent complex and supports the final proteasome maturation (Fig 5C). Such a POMP-mediated model allows for efficient organization of the assembly machinery and the concerted arrangement of this heteromultimeric complex. At

the same time, this mechanism would deliver a sufficient amount of both subtypes of *de novo*-formed proteasomes (c20S and i20S), the function of which is required at the ER for protein quality control as well as for the generation of MHC class I-restricted antigenic peptides.

METHODS

Immunostaining. Cells were grown on slides, fixed (3.7% paraformaldehyde), permeabilized (0.2% Triton X-100), stained with antibodies against LMP2, LMP7, 20S (mAb; all laboratory stock), POMP (Witt *et al*, 2000), AbC8 (Nandi *et al*, 1997), calnexin (mAb; BD Biosciences, Franklin Lakes, NJ, USA) or ERAP1 (mAb; Saveanu *et al*, 2005) and processed with Alexa488- or Alexa568-conjugated (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) secondary antibodies. Images were captured with a Leica TCS SP2 DM LFSA confocal microscope system using standard filters and the respective software.

Flotation of microsomes. HeLa cells were Dounce homogenized in 10 mM HEPES (pH 7.2), 5 mM MgAc, 80 mM KAc, 5 mM $MgCl_2$, complete EDTA free (Roche Diagnostics, Mannheim, Germany), 10 μM MG132 and 0.5 M sucrose. The sucrose concentration was raised to 2.0 M and the mixture was centrifuged in a sucrose gradient (2.0–1.0 M) for 6 h at 100,000*g* and fractionated. See the supplementary information online for further details.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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