Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope–ER network in yeast

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26S proteasomes are the key enzyme complexes responsible for selective turnover of short-lived and misfolded proteins. Based on the assumption that they are dispersed over the nucleoplasm and cytoplasm in all eukaryotic cells, we wanted to determine the subcellular distribution of 26S proteasomes in living yeast cells. For this purpose, we generated yeast strains that express functional green fluorescent protein (GFP) fusions of proteasomal subunits. An α subunit of the proteolytically active 20S core complex of the 26S proteasome, Pre6/YOL038w, as well as an ATPase-type subunit of the regulatory 19S cap complex, Cim5/YOL145w, were tagged with GFP. Both chimeras were shown to be incorporated completely into active 26S proteasomes. Microscopic analysis revealed that GFP-labelled 20S as well as 19S subunits are accumulated mainly in the nuclear envelope (NE)–endoplasmic reticulum (ER) network in yeast. These findings were supported by the co-localization and co-enrichment of 26S proteasomes with NE–ER marker proteins. A major location of proteasomal peptide cleavage activity was visualized in the NE–ER network, indicating that proteasomal degradation takes place mainly in this subcellular compartment in yeast.

Keywords: ER network/GFP/NE/yeast 26S proteasome

Introduction

Metabolic and transcriptional control mechanisms, signal transduction and cell cycle progression depend on specific regulatory mechanisms including protein breakdown. The central enzyme complexes responsible for selective proteolysis of short-lived proteins are 26S proteasomes. Proteasomal substrates include metabolic key enzymes, transcription factors and cyclins, that are found to be targeted to proteasomal breakdown by conjugation to ubiquitin (Hershko and Ciechanover, 1992; Hochstrasser, 1995; Coux *et al.*, 1996; Hilt and Wolf, 1996).

The 26S proteasome consists of a proteolytically active 20S core complex and a regulatory 19S cap complex. In the yeast *Saccharomyces cerevisiae*, the subunit arrangement of the 20S core complex has been resolved by X-ray crystallography (Groll *et al.*, 1997). Fourteen different subunits, classified into seven α - and seven β -type subunits, are arranged in four heptameric rings stacked into

a hollow cylinder. Both outer rings contain the α -type subunits, both inner rings the β -type subunits. The interior of the 20S particle harbours the catalytic centres of the protease. N-terminal threonine residues of certain β -type subunits form the central active sites that are liberated by precursor processing during proteasome assembly (Chen and Hochstrasser, 1996; Schmidtke *et al.*, 1996; Seemueller *et al.*, 1996; Heinemeyer *et al.*, 1997). Different peptide cleavage preferences could be assigned to distinct β -type subunits by using yeast genetics (Hilt *et al.*, 1994; Arendt and Hochstrasser, 1997).

The regulatory 19S cap complex of the 26S proteasome confers the specificity towards ubiquitinated substrates and an ATP dependence on proteolysis (Dubiel *et al.*, 1995; Tanaka and Tsurumi, 1997). In yeast, the 19S cap complex comprises six AAA-type ATPases and 11 non-ATPases (Glickman *et al.*, 1998). The ATPase subunits were proposed to function as reverse chaperones in substrate unfolding and translocation into the proteolytic cavity of the proteasome (Confalonieri and Duguet, 1995). Ubiquitin binding and isopeptidase activities were assigned to non-ATPase subunits, responsible for editing and rescuing ubiquitin moieties from the substrate prior to degradation (Deveraux *et al.*, 1994; Lam *et al.*, 1997).

Different genetic approaches using yeast mutants deficient in degradation of short-lived proteins or defective in cell cycle progression identified several 26S components and provided considerable insight into the *in vivo* functions of 26S proteasomes. In particular, the timely progression of the cell cycle turned out to be tightly regulated by selective proteolysis through proteasomes (Ghislain *et al.*, 1993; Gordon *et al.*, 1993; Friedman and Snyder, 1994; Kominami *et al.*, 1995, 1997; Hilt and Wolf, 1996; McDonald and Byers, 1997; Wilkinson *et al.*, 1997).

Genetic studies in yeast further revealed that the ubiquitin–proteasome machinery is also involved in endoplasmic reticulum (ER) degradation (Hampton *et al.*, 1996; Hiller *et al.*, 1996). A retrograde transport mediated by ER translocons and the ER-resident chaperone Kar2/Bip is required to transport abnormal proteins from the ER lumen to the cytoplasmic face for final proteolysis (Plempner *et al.*, 1997).

In higher eukaryotic cells, immunolocalization and biochemical fractionation studies showed a cytoplasmic and nucleoplasmic localization of proteasomes (Peters *et al.*, 1994). The amounts of nuclear proteasomes were found to be increased in mitotic cells (Amsterdam *et al.*, 1993; Palmer *et al.*, 1996), particularly in highly proliferating cells during early stages of *Drosophila* embryogenesis, during *Xenopus* oocyte maturation and in tumorigenic cells (Klein *et al.*, 1990). In human fibrosarcoma cells, a subpopulation of green fluorescent protein (GFP)-tagged proteasomes was shown to be taken up mainly into the nucleus upon the reassembly of the nuclear membrane

during mitosis, but was also reported to be transported unidirectionally over the nuclear membrane (Reits *et al.*, 1997).

In order to study *in vivo* the subcellular distribution of yeast 26S proteasomes, we tagged the Pre6/YOL038w α -type subunit of the 20S core complex (Heinemeyer *et al.*, 1994) as well as the Cim5/YKL145w ATPase-type subunit of the 19S regulatory complex (Ghislain *et al.*, 1993) with GFP fused to a double hemagglutinin (HA) epitope. The GFP-labelled proteasome subunits were shown to be incorporated into active 26S proteasomes, thus providing suitable reporters of proteasomal degradation in living yeast cells. Using different techniques, proteasomes were observed to be enriched in the nuclear envelope (NE)-ER network independently of the cell and life cycle phases. Furthermore, the analysis of NE-ER fractions showed the majority of proteasomes to be bound to membrane structures. Proteasomal peptide cleavage occurred mainly in the NE-ER network, suggesting a major degradation location in this subcellular compartment in yeast.

Results

Generation of yeast strains expressing functional GFP-tagged proteasomes

In higher eukaryotes, 26S proteasomes are dispersed over the cytoplasm and nucleoplasm (Peters *et al.*, 1994; Reits *et al.*, 1997). In this work, we set out to study in detail the *in vivo* distribution of proteasomes in yeast. Our experimental approach was based on GFP technology, since GFP fusion proteins have become the tool of choice for visualizing molecular events *in vivo* by direct fluorescence microscopy (Cubitt *et al.*, 1995). To provide suitable reporters of the subcellular localization of 26S proteasomes in living cells, we generated yeast strains that express functional GFP-tagged proteasomal subunits. The α -type subunit Pre6/YOL038w of the outer rings (Heinemeyer *et al.*, 1994) was chosen to mark the proteolytically active 20S core complex of the proteasome. The ATPase-type subunit Cim5/YOL145w (Ghislain *et al.*, 1993) was selected to monitor the subcellular distribution of the regulatory 19S cap complex. Both Pre6 and Cim5 are encoded by single essential genes (Ghislain *et al.*, 1993; Heinemeyer *et al.*, 1994). To allow the expression of each GFP chimera under the control of its own promoter, the chromosomal copies of *PRE6* and *CIM5* were disrupted 5' to their stop codons by in-frame insertion of the GFP-coding region fused with a double HA epitope sequence. The chromosomal replacement of the wild-type genes by the corresponding GFP-HA-tagged versions was achieved by homologous recombination using *HIS3* and *URA3* genes as selection markers (Figure 1A). The *PRE6-GFP-HA::URA3::HIS3* and *CIM5-GFP-HA::URA3::HIS3* constructs were directed into the desired sites of the *PRE6* and *CIM5* locus upon transformation of wild-type strain WCGa, generating the yeast strains GCE6 and GAL5, respectively. Southern analysis of the chromosomal DNA of these strains confirmed that the original chromosomal copy was replaced by the respective GFP-HA-tagged version of the proteasomal subunit, including the flanking *URA3* and *HIS3* genes (data not shown).

Since single essential genes (Ghislain *et al.*, 1993;

Fig. 1. (A) Pre6 and Cim5 were tagged by an in-frame insertion of GFP-HA into the genomic *PRE6* and *CIM5* genes, generating C-terminal fusions. Adjacent *HIS3* and *URA3* genes were used as selection markers upon transformation of wild-type strain WCGa. Desired sites for homologous recombination of *PRE6-GFP-HA::HIS3::URA3* and *CIM5-GFP-HA::HIS3::URA3* into the original chromosomal loci, respectively, are indicated by dotted crossovers. ATG, start; TAA, stop codon. (B) Wild-type Cim5 is replaced by functionally expressed Cim5-GFP-HA in GAL5 (*CIM5-GFP-HA::HIS3::URA3*) and Cim5-HA in HAL5 (*CIM5-HA::HIS3::URA3*) cells, respectively. Total cell extracts of strain WCGa (lane 1), GAL5 (lane 2) and HAL5 (lane 3), respectively, were subjected to SDS-PAGE and analysed by immunoblotting using anti-Cim5 (left panel), anti-HA mAb12CA5 (middle panel) and anti-GFP antibodies (right panel).

Heinemeyer *et al.*, 1994) were replaced by GFP-HA-tagged versions, Pre6-GFP-HA and Cim5-GFP-HA were expected to be functionally expressed. Both strains GCE6 and GAL5 grew at rates comparable with wild-type strain WCGa at 30 and 37°C (data not shown). In addition, a strain named HAL5 was constructed which was obtained upon transformation of wild-type strain WCGa with *CIM5-HA::HIS3::URA3* (see Materials and methods).

The replacement of endogenous Cim5 by Cim5-GFP-HA and Cim5-HA was analysed by comparative SDS-PAGE and immunoblot analysis of cell lysates of strain GAL5 harbouring *CIM5-GFP-HA::HIS3::URA3*, of strain HAL5 harbouring *CIM5-HA::HIS3::URA3* and of their parental strain WCGa by using anti-Cim5, anti-GFP and anti-HA antibodies. Cim5 was absent in total cell extracts of GAL5, but was replaced by the Cim5-GFP-HA chimera (expected molecular mass of 84 kDa) (Figure 1B, left panel, compare lanes 1 and 2). Likewise, in the HAL5 cell lysate, no band corresponding to endogenous Cim5 was detectable. Instead a protein band of slightly higher molecular mass (~3 kDa) as expected for double HA-tagged Cim5 was visible (Figure 1B, left panel, lane 3). The C-terminal HA epitopes of Cim5-GFP-HA and Cim5-HA, respectively, were recognized by mAb12CA5, while no protein was detected in the parental wild-type strain (Figure 1B, middle panel). The GFP moiety within Cim5-GFP-HA was identified by probing the same blot with

Fig. 2. (A) Pre6-GFP-HA and Cim5-GFP-HA are incorporated into proteolytically active 26S proteasomes. Extracts of GCE6 and GAL5 cells expressing GFP-HA-tagged Pre6 and Cim5, respectively, were subjected to 10–40% glycerol gradient ultracentrifugation. Fractions were collected from top to bottom of the gradient and assayed for peptide cleavage activity using the substrate Cbz-Leu-Leu-Glu-β-naphthylamide (dotted line). All fractions were analysed for protein contents (solid line). GFP fluorescence intensity was measured (broken line). All values are given as a percentage of the maximum value. Comparable profiles were obtained for GAL5 and GCE6 cell lysates fractionated under equivalent conditions, thus GCE6 stands for GAL5. (B) Protein samples of each fraction were run on SDS-PAGE followed by immunoblot analysis using anti-GFP antibodies (GAL5 fractions, upper panel; GCE6 fractions, lower panel). (C) Pre6-GFP-HA immunoprecipitates with proteasomal 20S subunits. GCE6 cell lysates were incubated with protein A-Sepharose beads. The resin was analysed as a control for unspecific binding by SDS-PAGE followed by immunoblotting (without mAb12CA5; lane 1). The pre-cleared lysate was incubated with protein A-Sepharose beads and mAb12CA5 antibody. The immunoprecipitated proteins (IP) were probed for Pre6-GFP-HA, Sc11, Pre4 and Pre2, respectively (with mAb12CA5; lane 2).

anti-GFP antibodies (Figure 1B, right panel). Taken together, the data demonstrate that wild-type Cim5 is indeed replaced by HA- and GFP-HA-tagged versions in HAL5 and GAL5 cells, respectively.

GCE6 cells harbouring chromosomal *PRE6-GFP-HA::HIS3::URA3* (Figure 1A) were found to express a GFP-HA chimera of the expected size corresponding to Pre6-GFP-HA (59 kDa; refer to Figure 2).

The fact that Pre6 and Cim5 were expressed in fusion with GFP was not sufficient to consider them as suitable

markers for proteolytically active 26S proteasomes. Two criteria had to be fulfilled further. First, the GFP-HA-tagged versions of both subunits have to be integrated completely into their proteasomal subcomplexes, since maverick GFP-HA-labelled subunits will lead to misinterpretations concerning the localization of proteasome complexes. Secondly, both GFP-tagged subunits will only report on the location of proteasomal degradation if they are fully assembled with matured, proteolytically active core complexes.

To examine whether Pre6-GFP-HA and Cim5-GFP-HA were incorporated into proteasome complexes, cell extracts of strain GCE6 and GAL5 were analysed in parallel by density gradient ultracentrifugation. All fractions were assayed for proteasomal peptide cleavage activity and GFP fluorescence intensity. Proteasomal activity was detected in the rapidly sedimenting fractions, peaking in fractions 14–15 (Figure 2A; shown for GCE6). The same profile was found for GAL5 fractions (data not shown). GFP fluorescence intensity was found to peak with proteasomal activity, providing evidence that the fluorescence signals can be traced back to proteolytically active proteasomes. In addition, the proteins of each fraction were resolved by SDS-PAGE and probed with anti-GFP antibodies. The GFP moieties of Pre6-GFP-HA and Cim5-GFP-HA were detected exclusively within those fractions that peaked with proteasomal activity and GFP fluorescence intensity (Figure 2B). No GFP-HA antigens could be detected in the more slowly migrating gradient fractions. Thus, Pre6-GFP-HA as well as Cim5-GFP-HA were mainly part of proteolytically active proteasomes rather than of precursor intermediates that are formed during the processing of several β-type subunit precursors while 20S core complexes assemble (Chen and Hochstrasser, 1996; Schmidtke *et al.*, 1996; Seemueller *et al.*, 1996; Heinemeyer *et al.*, 1997).

The incorporation of Pre6-GFP-HA into the mature 20S core complex was investigated further by co-immunoprecipitation experiments. For this purpose, the C-terminal HA epitope following the GFP in-frame moiety within Pre6-GFP-HA turned out to be suitable, since mAb12CA5 monoclonal antibody bound to protein A-Sepharose allowed the easy isolation of the GFP-HA chimera (Figure 2C). The immunoprecipitated proteins were separated by SDS-PAGE, blotted and probed for the GFP-HA chimera and interacting 20S subunits by using available antibodies (Figure 2C, lane 1, control without mAb12CA5; lane 2, mAb12CA5 immunoprecipitate). Pre6-GFP-HA (α4_{sc}; nomenclature according to Groll *et al.*, 1997) was found to be associated with the α-type subunit Sc11 (α1_{sc}; molecular mass of 29 kDa) and the matured forms of the β-type subunits Pre2 (β5_{sc}; 23 kDa) and Pre4 (β7_{sc}; 26 kDa) (Heinemeyer *et al.*, 1997), respectively. These data substantiated our conclusions from glycerol gradient fractionations that suggested that Pre6-GFP-HA is integrated into the active 20S core complex. Meanwhile, crystallographic structural analysis of 20S proteasomes predicted that the C-terminal region of Pre6 protrudes to the protein surface (Groll *et al.*, 1997), explaining the accessibility of the HA tag for the mAb12CA5 antibody.

Immunoprecipitation experiments using GAL5 cell lysates showed the interaction of Cim5-GFP-HA with proteasomal 20S subunits (data not shown) as expected for

Fig. 3. *In vivo* localization of GFP-tagged proteasomes in yeast. Cells of strain GCE6 and GAL5 expressing GFP-tagged versions of Pre6 and Cim5, respectively, were monitored by direct fluorescence microscopy (20S Pre6-GFP-HA, upper panels; 19S Cim5-GFP-HA, lower panels). The fluorescent proteasome subcomplexes were detected by using the FITC channel (GFP; left panels). Cells were viewed by Nomarski optics, and the DAPI-stained nuclei were visualized by simultaneously opening the UV channel (DIC/DAPI; right panels). Arrows indicate mitotic cells. Bar, 3 μ m.

a 26S subunit (Ghislain *et al.*, 1993). Thus, we concluded that the entire population of 26S proteasomes is confined by both Cim5-GFP-HA and Pre6-GFP-HA fusions.

Yeast 26S proteasomes are located mainly in the NE-ER network

The subcellular distribution of GFP-tagged 20S and 19S proteasome subcomplexes was monitored in living cells of strain GCE6 and GAL5, respectively (Figure 3). The cells were visualized by Nomarski optics; the 4',6-diamidino-2-phenylindole (DAPI)-stained nuclear DNA was highlighted by simultaneous UV excitation (right panels). Pre6-GFP-HA of the proteolytically active 20S core complex and Cim5-GFP-HA of the regulatory 19S complex of the 26S proteasome were found to be accumulated predominantly in the nuclear periphery (left panels). The coincident subcellular distribution of both proteasomal subunits indicated that 20S and 19S subcomplexes are associated with each other *in vivo*, thus demonstrating that

26S holocomplexes are located mainly in the nuclear periphery. An estimation of the GFP signals over the cross-section of a single yeast cell revealed that ~80% of 26S proteasomes are structurally bound to the nuclear periphery, while 20% of these complexes exist in the cyto- and nucleoplasmic compartments.

The pattern of GFP-labelled proteasomes strongly resembled those patterns that were reported for GFP-labelled nuclear pore ER membrane proteins (Belgareh and Doye, 1997; Bucci and Went, 1997). These observations gave rise to the question of whether proteasomes co-localize with marker proteins of the NE-ER network such as Kar2/Bip (Rose *et al.*, 1989). For indirect immunolocalization studies, HAL5 cells expressing the HA-tagged version of Cim5 instead of the wild-type subunit were preferred to GCE6 or GAL5 cells, because the intrinsic fluorescence of GFP-labelled proteasomes was drastically reduced during cell fixation. HAL5 cells were processed and probed for Cim5-HA (Figure 4A, upper left panel; visualized by Cy3-conjugated donkey anti-mouse IgG) and Kar2 [Figure 4A, lower left panel; visualized by fluorescein isothiocyanate (FITC)-labelled goat Fab fragments anti-rabbit IgG]. Using confocal laser scanning microscopy, an overlapping signal for the proteasomal 26S subunit and Kar2 was monitored (Figure 4A, lower right panel). In particular, the dotted pattern along the nuclear rim with few peripheral extensions, as already observed for the GFP-HA-tagged variants in living cells (Figure 3, left panels), could be resolved. Since the rough ER (RER) is scarcely distinguishable from the NE (Strambio-de-Castillia *et al.*, 1995), we also performed a double immunolocalization of proteasomes and nuclear pore membrane proteins. Diploid wild-type cells of strain WCGa/ α were probed with anti-20S proteasome antibodies (Figure 4B, upper panel; visualized by FITC-labelled goat Fab fragments anti-rabbit IgG) and mAb414 nuclear pore proteins (Figure 4B, middle panel; visualized by Cy3-conjugated donkey anti-mouse IgG) and stained with DAPI (Figure 4B, lower panel). Again, a coincident localization at the nuclear rim was observed, supporting our interpretation that proteasomes are accumulated in the NE-RER.

Our observation that proteasomes remained accumulated in the NE-ER in mitotic cells (marked by arrows in Figure 3) suggested that the majority of proteasomes persist at the nuclear rim throughout the cell cycle. Using α -factor and nocodazole, GCE6 cells were arrested at the G₁-S transition and released into M phase, respectively (Seufert *et al.*, 1995). As already shown for non-synchronized cells (Figure 3), proteasomes remained accumulated in the NE-ER independently of the cell cycle stage (data not shown).

Furthermore, we were interested in whether the subcellular distribution of GFP-tagged proteasomes is disturbed in proteasomal mutant strains *cim3* and *cim5* that are defective in cell cycle progression and arrest during mitosis (Ghislain *et al.*, 1993). Both strains were transformed with the *PRE6-GFP-HA::HIS3::URA3* construct, resulting in strains expressing Pre6-GFP-HA instead of wild-type Pre6 in a *cim3* and *cim5* mutant background, respectively. Even at the non-permissive temperature (37°C), no aberrant localization of GFP-labelled proteasomes could be observed (data not shown). This suggested that anaphase

promotion in *cim3* and *cim5* mutants is not delayed due to a mislocalization of proteasomes that might impair the turnover of mitotic cyclins required for proper cell cycle progression.

Proteins that are bound peripherally or integrally to the contiguous network of inner-outer NE-ER membranes can be distinguished further by using a membrane fusion assay established by Latterich and Schekman (1994). Their movements together with NE-ER membranes can be followed during zygote formation when the NE-ER bilayer of two conjugating cells joins and their contents intermix as

a consequence of nuclear fusion (Latterich and Schekman, 1994; Belgareh and Doye, 1997; Bucci and Wentle, 1997). If proteasomes are anchored to NE-ER membranes, they ought to be retained in these structures throughout the mating process. Therefore, GCE6 (*MATa*) and GCE6 (*MAT α*) cells, both expressing GFP-tagged proteasomes, were permitted to mate. Upon nuclear fusion, the majority of 26S proteasomes were observed to intersperse the intermixing NE-ER network of the premature diploid cell (Figure 5A). Structurally bound proteasomes finally were inherited by the budding-off daughter cell from the diploid mother cell, obviously by membrane fissions (Figure 5B).

Biochemical fractionation reveals co-enrichment of 26S proteasomes and NE-ER membrane proteins

Based on our localization studies, we expected 26S proteasomes to be enriched with NE-ER markers in biochemical fractionation experiments. To isolate NE-ER membranes from GCE6 cells, we applied a procedure based on the preparation of highly enriched yeast nuclei (Rout and Blobel, 1993; Strambio-de-Castillia *et al.*, 1995). By lysing these nuclei, NE-ER membranes were released and isolated by flotation in an equilibrium sedimentation gradient. Proteins of the enriched NE-ER fraction of the nuclear lysate and of total spheroplasts were resolved by SDS-PAGE and analysed by immunoblotting. As expected (Strambio-de-Castillia *et al.*, 1995), nucleoporins and the ER luminal marker Kar2 were co-enriched within the NE-ER membrane fraction (Figure 6A, compare lane 1 with lanes 2 and 3). Nucleolar Nop1 was almost undetectable in the NE-ER fraction, indicating only a small amount of nucleoplasmic remnants within this fraction. Compared with total cell lysates, proteasomal 26S subunits were found to be enriched with NE-ER membrane markers as analysed for Pre6-GFP-HA, Cim5 and Sc11, respectively.

To estimate the relative amounts of structurally bound proteasomes versus soluble proteasomes, spheroplasts of strain GCE6 were lysed by sonication, yielding >95% cell disintegration. The membranes were pelleted by ultracentrifugation and separated from the high speed supernatant. Cell equivalents of total lysate (Figure 6B, lane 1), high speed supernatant (Figure 6B, lane 2) and membrane resuspension (Figure 6B, lane 3) were probed for Kar2, Pre6-GFP-HA and Sc11, respectively. The luminal ER marker was almost completely removed from the high speed supernatant, indicating only a small amount

Fig. 4. (A) Co-localization of Cim5-HA and Kar2/Bip, a reporter protein of the NE-ER network, by confocal laser scanning microscopy. HAL5 cells were processed for indirect immunofluorescence and double-labelled with mAb12CA5 and anti-Kar2 antibodies followed by incubation with Cy3-labelled donkey anti-mouse IgG (Cim5-HA, upper left panel) and FITC-labelled goat Fab fragments anti-rabbit IgG (Kar2, lower left panel). Nuclear DNA was stained with DAPI (upper right panel). All three images were merged, revealing a co-localization within dot-like structures around the nuclear DNA (lower right panel; Bar, 2 μ m). (B) Double-labelling of proteasomes and mAb414 nuclear pore proteins in diploid WCGa/α cells. Fixed cells were probed with both anti-20S proteasome antibodies (Seeger *et al.*, 1996) followed by incubation with FITC-labelled goat Fab fragments anti-rabbit IgG (upper panel) and mAb414 antibodies (Rout and Blobel, 1993), and then by Cy3-labelled donkey anti-mouse IgG (middle panel). The DNA was stained with DAPI (lower panel).

Fig. 5. GFP-tagged proteasomes move along the NE-ER upon zygote formation. Diploid cells were formed by crossing haploid cells of strain GCE6 α and strain GCE6 α , both expressing fluorescent proteasomes. Cells were viewed by Nomarski optics (DIC, left panels) and the FITC channel (GFP, right panels). (A) While the NE-ER networks of the conjugating cells were joining, proteasomes were spread over the intermixing membranes. (B) The structurally bound proteasomes were inherited by the budding-off daughter cell from the new diploid mother cell through NE-ER membrane fissions.

of microsomal remnants in the soluble fraction. The proteasomal subunits Pre6-GFP-HA and Sc11 were found to be highly enriched within the membrane resuspension compared with the soluble fraction. To quantify the GFP signals, the fluorescence intensity of each fraction was measured. In the membrane resuspension, we determined $80 \pm 5\%$ of the total GFP labels, which is in good agreement with our estimations of structurally bound and soluble proteasomes in intact cells (Figure 3).

Proteasomal peptide cleavage occurs mainly in the NE-ER network in yeast

To visualize proteasomal activity in the NE-ER network, we employed an *in situ* test that allowed the selective screening for proteasomal mutants in previous work by taking advantage of the peptide cleavage ability of proteasomes in permeabilized yeast cells (Enenkel *et al.*, 1994). GCE6 cells were transferred into spheroplasts and embedded into a medium containing the proteasome-specific peptide substrate Cbz-Leu-Leu-Glu- β -naphthylamide. Fluorescence microscopy revealed a coincident staining of GFP-labelled proteasomes and released β -naphthylamine chromophores, finally confirming that a major part of proteasomal proteolysis indeed takes place in the NE-ER network in yeast (Figure 7).

Fig. 6. Yeast 26S proteasomes are enriched with NE-ER membrane fractions. (A) NE-ER membranes were prepared according to Strambio-de-Castillia *et al.* (1995). Fractions were analysed by SDS-PAGE followed by Coomassie Blue staining (upper panel) and immunoblotting (lower panel). Equivalent amounts of protein of total spheroplast lysates (T, lane 1), highly enriched nuclear lysates (N, lane 2) and NE-ER fractions (NE, lane 3) were resolved and probed for mAb414 nuclear pore membrane proteins, nucleolar Nop1 (Rout and Blobel, 1993), ER luminal Kar2/Bip (Rose *et al.*, 1989), the proteasomal subunits Pre6-GFP-HA, Cim5 and Sc11, and phosphofructokinase Pfk1/Pfk2, respectively. (B) The relative amounts of proteasomes either existing in the soluble fraction or bound to membrane structures were estimated. Total cell lysates were obtained by sonication of GCE6 spheroplasts. Membranes were pelleted by ultracentrifugation, separated from the high speed supernatant and resuspended. Cell equivalents of total lysate (T, lane 1), high speed supernatant (S, lane 2) and membrane pellet (P, lane 3) were analysed by SDS-PAGE and immunoblotting using anti-Kar2, GFP and Sc11 antibodies. Each fraction was assayed for relative GFP fluorescence intensity. The mean values given were derived from measurements of three parallel preparations.

Discussion

In higher eukaryotic cells, GFP-tagged 20S proteasomes were found to be dispersed equally over the cytoplasm and nucleoplasm and shown to be intermixed rapidly between these compartments during the breakdown of nuclear membranes during mitosis (Reits *et al.*, 1997).

The subcellular distribution of proteasomes was expected to be conserved in all eukaryotes from yeast to man. Contrary to all expectations, we found a significant accumulation of active core and regulatory complexes of

Fig. 7. *In situ* staining of yeast cells using the proteasome-specific substrate Cbz-Leu-Leu-Glu- β -naphthylamide revealed a major subcellular location of proteasomal degradation in the NE-ER network. Spheroplasts of strain GCE6 were embedded in sucrose medium containing 1% molten agar and 10 mM Cbz-Leu-Leu-Glu- β -naphthylamide. The embedding mixture was mounted directly on slides and monitored by fluorescence microscopy. The GFP-labelled proteasomes were visualized by the FITC channel (upper panel), and the proteasomal cleavage product β -naphthylamine (BNA) by the UV channel (lower panel).

the yeast 26S proteasome in the NE-ER network by using tagged variants of proteasomal subunits, as well as different localization and fractionation techniques. The coincident subcellular distribution of GFP-labelled Pre6 and Cim5 suggested that 19S and 20S subcomplexes are *in vivo* associated with each other, acting together as 26S holo-complexes. Independently of the cell cycle, proteasomes could be localized mainly in the NE-ER. Their membrane association could be demonstrated further by exploiting a special aspect of the yeast life cycle. Upon mating, NE-ER bilayers of two conjugating cells join and intermix their contents, while the fate of either a peripheral or integral membrane protein can be monitored (Latterich and Schekman, 1994). This assay demonstrated that proteasomes remain in the intermixing NE-ER network throughout zygote formation and are passed on from mother to daughter cells by membrane fissions, providing further evidence that proteasomes physically interact with these structures.

Confocal laser scanning microscopy revealed an over-

lapping localization of proteasomal subunit Cim5 and Kar2 especially around the nuclear DNA. The double immunofluorescence of proteasomes and nuclear pore membrane proteins provided further evidence that proteasomes are accumulated in the NE-ER.

The only previous publication on yeast proteasome localization describes two 19S subunits, Sen3 and Pcs1/Sug2, as residing within the nucleoplasm, based on indirect immunofluorescence (McDonald and Byers, 1997). Even though these data seem to differ from ours, both localizations overlap at least at the NE double membrane. The different interpretations of the localization studies might be due to the fixation conditions that were applied for indirect immunofluorescence. Subcellular distributions may also vary with the expression level of the respective proteasomal subunits. The existence of 19S subunits inside the nucleoplasm might indicate that regulatory components of the 26S proteasome are only partly associated with proteasome core complexes (Russell *et al.*, 1996; McDonald and Byers, 1997). This view is supported by data of several groups which show that 19S regulatory components exist in multiple complex organizations or as free subpopulations, that are required for specific nuclear functions (Russell *et al.*, 1996; Fraser *et al.*, 1997).

Recently, VCP, an abundant protein complex that belongs, like several 19S cap subunits, to the AAA-type ATPase family, was shown to mediate transport of an ubiquitinated substrate to the ubiquitin-proteasome degradation pathway (Dai *et al.*, 1998). Since the yeast homologue of VCP, Cdc48, is located mainly in the NE-ER network (Latterich *et al.*, 1995), Cdc48/VCP might be a potent chaperone that provides another link between substrate unfolding and translocation to proteasomal degradation.

A distinct population of proteasomes, not restricted to yeast, was found to be ER associated (Palmer *et al.*, 1996). Also, 19S homologous complexes involved in signal transduction of higher eukaryotic cells recently were described as residing in the NE periphery (Seeger *et al.*, 1998).

Our cytological results were reflected in biochemical fractionation experiments. In support of this, proteasomal peptide cleavage activity was detected mainly in the NE-ER network. These findings suggest that a major location of substrate turnover is at the NE-ER network in yeast. Thus, proteasomal turnover might be regulated by subcellular compartmentalization, with the consequence that a major group of proteins destined for degradation may be recruited to the NE-ER network. Concerning short-lived nuclear proteins destined for degradation, one may imagine that they are targeted from the nucleoplasm to the NE-ER upon conjugation to ubiquitin. Alternatively, ubiquitination of short-lived proteins may occur directly in the NE-ER as proposed by Biederer *et al.* (1997). They showed that a soluble ubiquitin-conjugating enzyme, Ubc7, only participated in the turnover of soluble proteins after binding to the NE-ER surface, a process that was found to be mediated by Cue1 receptors. In this scenario, substrates are delivered to the ubiquitin-proteasome degradation machinery right at the NE-ER. Consistent with this model is the finding that soluble substrates tagged with hydrophobic extensions are only metabolically stable in *ubc6 ubc7* mutants, suggesting that hydrophobic

Table I. Nucleotide sequences of primers used in this study

Gene	Primer	Nucleotide sequence
<i>PRE6</i>	P1	5'-AAAAGCATGCGGCCGCACAAATGAGTGGTTACGAT-3' <i>SphI</i> – <i>NotI</i>
	P2	5'-AAAAGGTACCCGGATCCATGGTTAGATTTTTCTTTTGTGTC-3' <i>BamHI</i> – <i>KpnI</i>
	P3	5'-AAAAGAATTCGCGCATATATATGGGC-3' <i>EcoRI</i>
	P4	5'-AAAACTCGAGTGTCCATGACAAACT-3' <i>XhoI</i>
<i>CIM5</i>	P1	5'-AAAAGTCGACGCGGCCGCATGCCACCAAAAGAAGACTGGG-3' <i>Sall</i> – <i>NotI</i>
	P2	5'-AAAAGGATCCATTATATTGCATATAACGCG-3' <i>BamHI</i>
	P3	5'-AAAAGAATTCCTGACGTCAATGTACTTTTITA-3' <i>EcoRI</i>
	P4	5'-AAAACTCGAGCCGTCGAGGAAGGCCAATT-3' <i>XhoI</i>

regions which are usually buried inside globular proteins or inserted into membranes are recognized preferentially as degradation signals by the ubiquitin–proteasome system (Gilon *et al.*, 1998).

Taken together, the presence of the majority of proteasomes in the NE–ER network indicates a local requirement for proteolytic elimination of protein substrates accumulating in this cellular compartment. However, not only ER and membrane proteins destined for degradation will meet the ubiquitin–proteasome degradation system here, once they are retranslocated out of the ER lumen to the cytoplasmic face (Hiller *et al.*, 1996; Plemper *et al.*, 1997). Nascent polypeptide chains that are not folded correctly might be co-translationally ubiquitinated (Sato *et al.*, 1998) and immediately turned over at the ER by proteasomal degradation. Proteasomes might indeed play a central role in quality control and co-translational processing of newly synthesized proteins (Lin *et al.*, 1998), thus assuming a key function not only in regulating protein degradation but also in protein synthesis.

NE–ER network association of proteasomes might be connected further with the final maturation of these multi-subunit complexes. This raises questions with regard to possible targeting of proteasomes to NE–ER membranes, and suggests the presence of novel regulatory mechanisms for proteasome functions.

Materials and methods

Plasmids and strains

Molecular cloning, yeast genetics and manipulation were performed following standard procedures (Guthrie and Fink, 1991; Ausubel *et al.*, 1995). GCE6 (*MATa his3-11,15 leu2-3,112 ura3 PRE6-GFPHA::HIS3::URA3*), GAL5 (*MATa his3-11,15 leu2-3,112 ura3 CIM5-GFPHA::HIS3::URA3*) and HAL5 (*MATa his3-11,15 leu2-3,112 ura3 CIM5-HA::HIS3::URA3*) are derived from WCGa (*MATa his3-11,15 leu2-3,112 ura3 can GAL*; Heinemeyer *et al.*, 1994), and GCE6 α is derived from WCG α . The strains were created by integrative transformation of the codons for GFP-HA, flanked by the *HIS3* and *URA3* genes at the 3' end

of the respective open reading frame using homologous recombination techniques.

PRE6 codons 1–254 were PCR-amplified using primers P1 and P2. P2 deleted the stop codon and incorporated a *BamHI* and a *KpnI* site in-frame. The PCR product P1P2 was digested with *SphI*–*KpnI* and ligated into pJD325 (kindly provided by Erica Johnson, HHMI, Rockefeller University, New York, NY, and Jürgen Dohmen, Heinrich Heine Universität, Düsseldorf, Germany), resulting in plasmid pPRE6HA. pPRE6HA contained in-frame with the *KpnI* site a nucleotide sequence of a double HA epitope followed by a stop codon. From this plasmid, a *Sall*–*XbaI* fragment containing *PRE6* codons 132–254 and the C-terminal HA tag was excised and ligated into pBluescript. The resulting plasmid pBSHA contained a single *BamHI* site following the penultimate codon of *PRE6*. Into that site, a *BamHI* fragment encoding the GFP S65→T variant (kindly provided by Steffen Rupp, White Head Institute, MIT, Boston, MA) was inserted, yielding pBSC6GFPHA. The *PRE6* downstream region was PCR-amplified using primers P3 and P4. The PCR product P3P4 was inserted as an *EcoRI*–*XhoI* fragment into the vector pBSHU, that was derived from pBluescript containing, between the polylinker sites *BamHI* and *SmaI*, the *HIS3* gene excised as a *BamHI*–*PstI* fragment from pJJ215 and the *URA3* gene excised as an *NsiI*–*PvuII* fragment from pJJ244 (Jones and Prakash, 1990). Into the resulting plasmid that contained the *HIS3* and *URA3* markers flanked by the downstream region of *PRE6*, *PRE6*-HA, which was obtained as a *NotI*–*XbaI* fragment from pPRE6HA, was inserted. This yielded pBS-PRE6HA-HU. Within pBS-PRE6HA-HU, codons 132–254 fused to HA were excised by *Sall*–*XbaI* and replaced by the GFP-HA-tagged version that was obtained as a *Sall*–*XbaI* fragment from pBSC6GFPHA. The final plasmid was named pBS-PRE6-GFPHA-HU. The DNA stretch containing *PRE6*-GFPHA flanked by *HIS3*, *URA3* and further downstream regions of *PRE6* was excised as a *Sall*–*XhoI* fragment from pBS-PRE6-GFPHA-HU and transformed into WCGa and α , respectively, using Ura⁺ His⁺ double selection. The chromosomal DNA of the Ura⁺ His⁺ transformants was analysed by Southern blot hybridization and PCR.

To generate tagged versions of Cim5, the PCR product P1P2 was inserted as a *Sall*–*BamHI* fragment into pBSHA, resulting in pBS-CIM5HA. Ligation of the *BamHI* fragment encoding GFP into pBS-CIM5HA yielded pBS-CIM5-GFPHA. The *CIM5* downstream region was PCR-amplified using primers P3 and P4, and the PCR fragment was cloned into pBSHU as described above. Into the resulting plasmid the DNA stretches containing *CIM5*-HA and *CIM5*-GFPHA that were excised as *NotI*–*XbaI* fragments from pBS-CIM5HA and pBS-CIM5-GFPHA, respectively, were cloned, yielding the final plasmids pBS-CIM5HA-HU and pBS-CIM5-GFPHA-HU. The DNA stretches con-

taining *CIM5-HA::HIS3::URA3* or *CIM5-GFP::HIS3::URA3* were transformed as *NotI-XhoI* fragments into WCGa. Ura⁺ His⁺ clones were selected and processed as above.

The nucleotide sequences of primers with restriction sites used for molecular cloning are listed in Table I.

Protein fractionation and immunoprecipitation

Yeast cells were grown to mid-log phase in YPD medium at 28°C and disintegrated using a French pressure cell and proteasome buffer (PB; 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol). After removal of cell debris (15 000 g, 20 min), the cell extracts were loaded onto 10–40% glycerol gradients made in PB. The samples were centrifuged for 16 h at 40 000 r.p.m. in a Sorvall TH-641 rotor at 4°C. Twenty fractions (0.6 ml) were collected, and aliquots were assayed for peptide hydrolysis activity against the substrate Cbz-Leu-Leu-Glu-β-naphthylamide (Enenkel *et al.*, 1994) and GFP-HA antigens. Protein contents were determined by using Bradford solution (Bio-Rad).

All fractions were scanned fluorescence intensity by using a spectrofluorophotometer (model RF-1501, Shimadzu, Kyoto, Japan; excitation, 490 nm; emission, 510 nm). To distinguish GFP fluorescence from autofluorescence, WCGa cell lysates were fractionated under the same conditions. The fractions obtained were scanned in parallel for autofluorescence. The resulting values defined the background fluorescence to which the values of the corresponding GFP fractions were referred.

Immunoprecipitations of GFP-HA fusion proteins were performed in the presences of bovine serum albumin (BSA; 0.1%, final concentration). Two ml of cell extract (5 mg/ml protein) were pre-cleared by overnight incubation with 50 µl of protein A-Sepharose beads (Pharmacia) at 4°C. The beads were removed by centrifugation (2000 g, 2 min), washed three times and boiled in non-reducing sample buffer. The pre-cleared cell extract was incubated with 10 µl (4 µg protein) of mAb12CA5 (Boehringer Mannheim, Germany) overnight. To separate the HA immunoprecipitates, 50 µl of protein A-Sepharose beads were added for 1 h at 4°C, sedimented by centrifugation, washed three times and boiled in non-reducing sample buffer. Proteins bound to the beads were identified by SDS-PAGE followed by immunoblotting. Antibodies were diluted in phosphate-buffered saline-Tris (PBST) containing 5% milk and detected by secondary horseradish peroxidase (HRP)-conjugated antibodies and ECL (Amersham). GFP was identified by anti-GFP polyclonals (Clontech, Palo Alto, CA), and proteasomal subunits by antibodies raised against Sc11, Pre2, Pre4 (Heinemeyer *et al.*, 1997) and Cim5 (Ghislain *et al.*, 1993), respectively. Anti-phosphofructokinase antibodies were a kind gift of Christel Gietz (Institut of Biochemistry, Charité, Berlin).

NE preparation

Yeast nuclear envelopes were prepared from 7 l of a log-grown cell culture of GCE6a (OD₆₀₀ 1–2) according to a protocol described by Strambio-de-Castillia *et al.* (1995). Cells were harvested and converted to spheroplasts in 1.1 M sorbitol using 1% (w/v) zymolyase 20 T (Seikagaku, Tokyo, Japan) and 10% (v/v) glucuronidase (DuPont NEN, Boston, MA). The spheroplasts resuspended in 1 vol. of 1.1 M sorbitol were divided in half, overlaid onto 15 ml cushions of 7.5% Ficoll in 1.1 M sorbitol and spun in a swinging bucket centrifuge (Beckman JS 13.1, 3000 g, 10 min). The sedimented spheroplasts were lysed in 30 ml of PVP (8% polyvinylpyrrolidone, 20 mM KPi pH 6.5, 1 mM MgCl₂, Boehringer Mannheim protease inhibitor mix) by several strokes with a smooth-fitting douncer on ice. Immediately, 1 vol. of 0.6 M sucrose in PVP was added and the crude nuclei were separated by centrifugation (13 000 g, 10 min). The crude nuclei were resuspended in 12 ml of 1.7 M sucrose in PVP, and the suspension adjusted with 14 ml of 2.3 M sucrose in PVP to a refractive index (RI) of 1.4250. Approximately 15 ml were overlaid over a three-step sucrose/PVP gradient [8 ml each of 2.01 M sucrose (RI = 1.4370), 2.10 M sucrose (RI = 1.442), 2.30 M sucrose (RI = 1.45) in PVP]. The gradients were run in a Beckman SW28 rotor at 28 000 r.p.m. for 4 h at 4°C. A dense white band at the 2.10–2.30 interface contained the bulk of the nuclei. The nuclear fraction was collected, adjusted to an RI of 1.434 with PVP and centrifuged at 45 000 r.p.m. for 1 h at 4°C (Beckman Ultracentrifuge, TLA45). The supernatant was removed by aspiration. The pellet containing highly enriched nuclei was resuspended in 3.33 ml of bt-DMSO [10 mM bis-Tris-HCl, pH 6.5, 0.1 mM MgCl₂ containing 20% (v/v) dimethylsulfoxide] in the presence of 20 µg/ml DNase I (Boehringer Mannheim) and incubated at room temperature for 10 min. The nuclear lysate was mixed with 10 ml of 2.1 M sucrose in bt containing 20% Nycodenz (Sigma) in a Beckman SW28 tube and overlaid with 12 ml of 2.0 M sucrose (RI = 1.4295) and 12 ml of 1.5 M sucrose (RI = 1.4055) in bt

buffer containing Boehringer Mannheim protease inhibitor mix. After centrifugation at 28 000 r.p.m. for 24 h at 4°C, the NE appeared as a fluffy band at the 1.5–2.0 interface. The NE fraction was collected, and samples were prepared for SDS-PAGE and immunoblot analysis.

For quantification, spheroplasts were lysed in 0.25 M sucrose-PB including protease inhibitors by sonication. Cells were counted before and after disintegration to ensure that >95% of the cells were disrupted. The lysate was cleared by ultracentrifugation and separated from the membrane pellet (Beckman TLA 45, 45 000 r.p.m., 1 h). The membranes were separated from the high speed supernatant and resuspended in 0.25 M sucrose-PB by sonication.

Localization studies

Immunofluorescence microscopy on formaldehyde-fixed cells was performed according to Strambio-de-Castillia *et al.* (1995). Double-labelling with mAb12CA5 (1:500 dilution)/anti-Kar2 antibodies (1:1000 dilution) and mAb414 (1:500 dilution)/affinity-purified anti-20S proteasome IgGs (1:100) was visualized by Cy3-labelled donkey anti-mouse IgG (cross-absorbed against rabbit IgG; 1:100 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) and FITC-labelled goat Fab fragments anti-rabbit IgG (no cross-reaction with mouse IgG; 1:500 dilution, Sigma).

To visualize nuclei in living cells, cells were briefly washed with 30% (v/v) methanol and incubated with 50 ng/ml DAPI in PBS for 5 min. The cells were washed with PBS and examined directly by fluorescence microscopy. To form zygotes, early-log phase cells of strains GCE6a and GCE6α were mixed and spread on YPD plates. Mating was carried out for 6–9 h at 28°C. The mating mixture was washed off the plates with SC medium and analysed by fluorescence microscopy.

Cells were visualized with a microscope (model DMR; Leica, Bensheim, Germany) equipped with a mercury lamp (HBO 50W, Osram) and a ×100/1.4 oil-immersion objective lens (PL Fluotar). To monitor the GFP fluorescence (excitation: 490 nm, emission: 510/540 nm) in living cells, the FITC channel was used. The DAPI-stained nuclei were visualized by the UV channel, the cells by Nomarski optics. Cells were exposed for the same length of time while micrographs were taken with a black and white cooled CCD camera (model C 5985-10, Hamamatsu, Hersching, Germany) connected to an Adobe Photoshop plug-in shutter and a Power Mac Computer. Images were printed on Epson photo quality glossy paper using an Epson Color Stylus 800 printer. To quantify GFP fluorescence, the exposure times were reduced to brightness values of a maximal 70% (Adobe Photoshop 4.0, level dialog box, image histogram), ensuring that light intensity and pixel brightness are on a linear scale. Using the eyedropper button, the brightness values within the nuclear periphery were ~20-fold higher than in the cytoplasm. Since the area of the nuclear periphery comprises about one-fifth of the whole cell section, 80% of GFP labels were calculated to reside within the nuclear periphery. The formation of zygotes was documented by a DMRD camera system using Kodak Elite 400 film. Coloured slides were processed for AGFA digiprints.

For *in situ* staining of peptide cleavage activity, spheroplasts were embedded in 0.6 M sucrose-PB containing 1% molten Bacto agar (Difco) and 10 mM Cbz-Leu-Leu-Glu-β-naphthylamide (Bachem, Switzerland). The mixture was poured onto microscope slides, covered and incubated at 25°C.

Confocal laser scanning microscopy was performed on a LSM 510 (Zeiss, Jena, Germany). Scan mode, plane; objective, Plan-Neofluar 100×/oil iris; scaling, X, Y: 0.04 µm; stack size, X, Y: 21.1 µm; scan zoom, 4.4; pixel time, 1.79 µs; wavelength, 543 nm, 364 nm, 488 nm.

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