Published in final edited form as:
Mech Ageing Dev. 2010 February ; 131(2): 144–155. doi:10.1016/j.mad.2010.01.002.

Molecular mechanisms of proteasome plasticity in aging

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Abstract

The ubiquitin-proteasome pathway plays a crucial role in regulation of intracellular protein turnover. Proteasome, the central protease of the pathway, encompasses multisubunit assemblies sharing a common catalytic core supplemented by regulatory modules and localizing to different subcellular compartments. To better comprehend age-related functions of the proteasome we surveyed content, composition and catalytic properties of the enzyme in cytosolic, microsomal and nuclear fractions. obtained from mouse livers subjected to organismal aging. We found that during aging subunit composition and subcellular distribution of proteasomes changed without substantial alterations in the total level of core complexes. We observed that the general decline in proteasomes functions was limited to nuclear and cytosolic compartments. Surprisingly, the observed changes in activity and specificity were linked to the amount of the activator module and distinct composition of the catalytic subunits. In contrast, activity, specificity and composition of the microsomal-associated proteasomes remained mostly unaffected by aging; however their relative contribution to the total activity was substantially elevated. Unexpectedly, the nuclear proteasomes were affected most profoundly by aging possibly triggering significant changes in cellular signaling and transcription. Collectively, the data indicate an age-related refocusing of proteasome from the compartment specific functions towards general protein maintenance.

Keywords

Proteasome; aging; mouse; liver; compartmentalization

1. Introduction

The proteasome is a multi-subunit and multifunctional protease that constitutes a part of the Ubiquitin Proteasome Pathway (UPP) (Glickman and Ciechanover, 2002). In the process of intracellular regulated proteolysis, proteasomes recognize, prepare and cleave substrates marked for degradation by enzymatic attachment of multiple ubiquitin molecules (Ub). The proteasome is essential and abundant enzyme in all eukaryotic cells (Groll et al., 2005; Hanna and Finley, 2007). This large protein complex is responsible for the controlled cleavage of most of the short-lived proteins in the cell, as well as for degradation of misfolded or oxidatively

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Contributors: KR conducted the experiments, did the initial analysis, proposed the initial draft of the manuscript, and contributed to the writing. MG analyzed the Western blots data and contributed significantly to the writing. PAO conducted the statistical analysis, and secondary analysis, developed the final figures and contributed significantly to the writing.

damaged proteins (Jung and Grune, 2008). Subcellular distribution of proteasomes varies depending on tissue and cell cycle stage and likely other factors. In general, from 50 to 90% of proteasomes may reside in the cytosol, including assemblies localized on a cytosolic side of endoplasmic reticulum and co-purifying with intracellular membranes ("microsomal proteasomes"). The remaining fraction of proteasomes is confined to the nucleus (Drews et al., 2007; Rivett, 1998; Wojcik and DeMartino, 2003).

The structure and catalytic mechanism of proteasome have been extensively studied (Groll et al., 2005). The eukaryotic 20S **c**ore **p**article (CP, ~700 kDa) is composed of 28 subunits arranged into a cylinder built from four stacked heteroheptameric rings. Each of the two internal β rings contains three distinct catalytic subunits, forming six threonine-type catalytic centers in the inner chamber; whereas the two outer α rings form gated channels leading to the chamber. The CP is capable of degrading polypeptides and unfolded proteins by breaking peptide bonds on a carboxyl side of hydrophobic (chymotrypsin-like peptidase activity/Ch-L, β5 subunit), basic (trypsin-like activity/T-L, β2), and acidic (peptidylglutamyl peptide hydrolysing or caspase-like activity/PGPH, β1) residues. (Arendt and Hochstrasser, 1997). The three specificities are retained regardless the form of the core and additional attachments, however their relative contribution to the degradation patterns may change profoundly. For example, in the jawed vertebrates, the core exists in two forms, the 'housekeeping' and "immuno"-proteasome, the latter especially efficient in delivering antigenic peptides for MHC class I system. Each form contains a unique set of exchangeable catalytic subunits (β1 or β1i, etc.) rendering distinct substrate specificity of the proteasome (Rock et al., 2002). Additionally, both forms are regulated by the attachment of additional modules to the universal interface of α ring, enormously broadening structural and functional diversity of the modular particles (Hanna and Finley. 2007). One of such attachments is an 11S activator complex (**p**roteasome **a**ctivator/ **reg**ulator PA28/REG) of exchangeable subunits, either heteromeric α/β or homomeric γ (Rechsteiner and Hill, 2005). To gain a competence in recognition and processing of ubiquitinated substrates, the CP is decorated with at least one 19S **r**egulatory **p**article (RP, cap). Double-capped core is known as the 26S proteasome (~2500 kDa). The RP is built from a lid, mediating substrate uptake and deubiquitination, and a base, responsible for substrate uptake and unfolding. The former is a complex of at least 10 Rpn subunits (**r**egulatory **p**article **n**on-ATPases), and the latter consists of six ATPases (Rpt1–6; **r**egulatory **p**article A**T**Pases), Rpn1 and 2, and a "hinge" Rpn10 (Hanna and Finley, 2007).

Studies implicating the role of the proteasome in aging are impressive in number but focused in scope and therefore difficult to integrate into the global understanding of the UPP physiology. For example, Ch-L and T-L activities have been reported to either increase (Shibatani, et al., 1996) not change (Anselmi et al., 1998; Conconi and Friguet, 1997) or decrease (Hayashi and Goto, 1998; Ferrington et al., 2005) with age both in rat liver and mouse muscle. PGPH activity has shown a decrease (Anselmi et al., 1998) or no change (Ferrington et al., 2005) with age. Each of the studies yields valuable information, however not straightforward to compare due to profound differences in methods and approaches. Other notable data include a reported age-related increase in immunoproteasome in rat skeletal muscle (Ferrington et al., 2005). Apart from direct measurements of protein content or enzymatic activity, studies on mRNA in gastrocnemius muscle revealed constant levels of core alpha subunits (Lee et al., 1999). and a decline of two catalytic subunits (β5i and β2). Most of studies are focusing on core particle, with the exception of RP analysis in *Drosophila* aging model and in desminopathy mouse hearts (Liu et al., 2006; Vernace et al., 2007).

One consequence of aging appears to be an increase in the incidence of lifespan-reducing liver pathologies. Aging in the liver is accompanied by a diminished volume, an increase in hepatocyte lipofuscin, and a loss of smooth surfaced endoplasmic reticulum. Also, the rate of liver regeneration following injury is decreased in old animals and humans (Schumucker,

2005). These observations may suggest an unbalanced protein turnover and thus possibly a diminished capacity of the UPP system in aging liver.

To better understand the role of the UPP in aging we attempted a comprehensive analysis of structure and function of proteasome assemblies in mouse liver. We collected liver samples from *ad lib* populations of young (6 month-), middle-aged (12 month-), and old (24 month-old) mice (C57-BL6). Since there has been long-standing evidence that the proteasome performs specific duties based on subcellular location (Wojcik and DeMartino, 2003), the liver extracts were separated into cytosolic, microsomal, and nuclear fractions. Subsequently, to determine a biochemical signature characteristic for each of these fractions, we approximated with Western blotting the contents of proteasomal subunits representing distinct subassemblies. Then, we supplemented the proteomics data with specific activities of three proteasomal peptidases using model fluorogenic peptide substrates.

2. Materials and Methods

2.1 Mice

Six-, twelve-, and twenty-four month old mice (C57BL/6) were housed in a standard light/dark cycle and given standard mouse feed and water *ad libitum*. The animals were euthanized and the liver was excised and flash frozen in liquid nitrogen. They were then stored at −80°C until further use.

2.2 Preparation of Extracts

Liver extracts were separated into nuclear, cytosolic, and microsomal fractions using a modified Millipore Corp. procedure (2005). Briefly, the liver from a single animal was weighed and disrupted in a Dounce homogenizer in RSB buffer (10mM HEPES, pH 6.2, 10mM NaCl, 1.4 mM $MgCl_2$) with or without a cocktail of protease inhibitors (1mM PMSF, 1mM NaF, 1mM leupeptin, 1μg/ml aprotinin, 1mM pepstatin) at a weight-to-volume ratio of 1 gram of tissue to 1 mL of buffer. After twenty strokes, the homogenate was centrifuged at 2,500 ×g for 5 minutes at 4°C. The supernatant was collected and the pellet was re-homogenized and centrifuged again under the same conditions. The resulting supernatants were pooled and centrifuged at 13,000 ×g for 90 minutes (Ti70, Beckman Coulter, Fullerton, CA, USA). The collected supernatant was labeled as the *cytosolic* fraction. The pellet was washed again in RSB buffer, re-dissolved in RIPA buffer (10mM Tris, pH=7.4, 10mM NaCl, 5mM $MgCl_2$, and 1mM DTT with or without the protease inhibitor cocktail) and then mixed for 2 hours at 4°C on a rocker. Next, the material was centrifuged at 16,000 ×g for 10 minutes and the supernatant was designated as the *nuclear* fraction.

The pellet obtained in the ultracentrifugation step was resuspended in RIPA buffer, mixed for 2 hours on a rocker at 4°C followed by centrifugation at 10,000 ×g for 6 minutes. The resulting supernatant was labelled as the *microsomal* fraction. Protein concentration was measured in all the fractions with the Bradford Protein Assay (Pierce, Thermo Scientific, Rockford, IL, USA). The aliquoted samples were stored at −20 C until needed.

2.3 Peptidolytic Activity Assays

In each fraction, the total peptidolytic activity of all types of the proteasomal assemblies was determined with fluorogenic model peptide substrates specific for each of the three classes of active centers: succinyl-LeuLeuValTyr-7-amido-4-methylcoumarin (SucLLVY-MCA; ChT-L; chymotrypsin-like; cleavage after hydrophobic residues), butoxycarbonyl-LeuArgArg-MCA (Boc-LRR-MCA; T-L; trypsin-like; cleavage after basic residues), and carbobenzoxy-LeuLeuGlu-MCA (Z-LLE-MCA; PGPH; post-glutamyl peptide hydrolyzing activity, post-acidic). The substrates were obtained from Bachem (Bachem Americas, Inc, Torrance, CA,

USA) or Calbiochem (San Diego, CA, USA). It was assumed that the amount of released free MCA from the substrates was proportional to peptidase activity of the catalytic sites responsible for cleavage after hydrophobic, basic, and acidic residues, respectively. Activity was measured in the absence and presence of a saturating concentration (10nM) of adamantane-acetyl-(6-aminohexanoyl)$_3$ -(leucinyl)$_3$-vinyl-(methyl)-sulfone (Ada-(Ahx)$_3$-(Leu)$_3$-vinyl sulfone; Calbiochem), a proteasome-specific inhibitor blocking all three active centers. The difference between the activity determined in the absence and presence of the inhibitor was used as a measure of the peptidolytic activity of proteasome. Activity measurements were performed in 50 mM Tris/HCl buffer, pH 8.0 containing 100μM substrate (final concentration), in the 96-well plate format at 37°C (Fluoroskan Ascent, Thermo Scientific, Rockford, IL, USA) keeping equal amounts of protein per sample (Gaczynska and Osmulski, 2005). Specific peptidolytic activity of proteasome was presented by two ways: (1) as pmol of released MCA in 1 min per 1 μg of total protein in a fraction or (2) per 1 μg of α7 subunit. Concentration of α7 subunit was determined using the standard curve prepared from quantitation of Western blots of highly purified 20S proteasome of known concentration.

2.4 Western Blot analysis

A semi-dry method was used to electrophoretically transfer proteins fractionated in 12% or 7% SDS-PAGE to nitrocellulose membranes (Osmonics, Inc., Minnetonka, MN, USA). The membranes were probed with antibodies against the following proteasome subunits: α4, α7, β1 (Y), β2 (Z), β5 (X), LMP2 (β1i), MECL1 (β2i), LMP7 (β5i), β4, PA28 α, β and γ, Rpn2, Rpn7, Rpn10, and Rpt5 (BioMol, Enzo Life Sciences, Plymouth Meeting, PA, USA). To test the purity of the subcellular fractions, compartment-specific antibodies to nuclear lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cytosolic GAPDH (Chemicon, Millipore Inc., Billerica, MA, USA), and microsomal calreticulin (Chemicon) were used. HRP-conjugated secondary antibodies were used to visualize the immuno-reaction using chemiluminescent substrate (Pierce). Immunoblots were quantified by densitometric analysis using Kodak Image Station 2000R imaging system and 1D gel analysis software v 3.6.2 (Kodak, San Diego, CA, USA). Content of the subunits expressed in arbitrary units per μg protein in the lysates was standardized against the know amount of a protein A conjugates (molecular weight standards, Chemicon) loaded on each gel.

2.5 Statistical Analysis

Livers from seven animals per each age group were analyzed. All the data were normalized prior to statistical analysis. Descriptive statistics was carried out with SPSS 14.0. To estimate variance homogeneity the Levene's test was applied. Since a positive result of this test was expected, we performed the *post hoc* Dunnett's or Tukey's tests for pairwise comparison of unequal or equal variances, respectively. A negative result of the Levene's test prompted the use of nonparametric tests for the group comparison. One-way ANOVA was used to compare age groups variance. Cluster analysis was performed using ChipSTC v 1.18 shareware (Peterson, 2006), Cluster 3.0 (Eisen at al., 1998, de Hoon et al., 2004) with TreeView 2.11 (Saldanha, 2004) and SPSS 14.0. Statistical significance was set at the $p < 0.05$ level.

3. Results

We confirmed with the compartment specific antibodies successful separation of cell lysates into cytosolic, microsomal and nuclear fractions. Each subcellular fraction was free of detectable protein markers of other fractions. Only a negligible amount of GADPH responsive material was found in a microsomal fraction obtained from livers of young mice (Fig. 1).

3.1 Age associated Changes in Total Peptidolytic Activity are fraction specific

In each subcellular compartment, peptidolytic activity of proteasome was measured with fluorogenic peptide substrates specific for each of three catalytic sites. In the separated subcellular fractions, the utilized peptides are also likely cleaved by other proteases. Therefore, to distinguish between proteasomal and total proteolytic activity toward the selected substrates, the assays were performed in the absence and presence of the irreversible proteasome inhibitor Ada-$(Ahx)_3$-$(Leu)_3$-VS that at the applied concentration blocks all three active sites (Gaczynska and Osmulski, 2005). The difference between these two measurements constitutes the combined peptidase activity exhibited by all types of proteasomal complexes. To estimate proteolytic challenge facing proteasome we expressed its specific peptidase activity as a reaction rate of MCA released (pmoles/min at 37°C) per μgram of total protein.

Fig. 2a shows that the sum of total specific activities (specific activity multiplied by fraction volume) of all peptidolytic sites (ChT-L+T-L+PGPH) in all the fractions (cytosolic +microsomal+nuclear) declined by about 40% in 12 and 24 mos when compared to 6 mos old animals. Fig. 2b shows that each fraction contributed differently to the decline. In the 6 mos group about a half of the total proteasomal activity was co-isolated with the microsomal fraction whereas one third was found in the cytosolic fraction and the remaining (about 7%) in the nuclear fraction. The partition of activity between the fractions changed dramatically with age. The share of microsomal fraction increased to 80% whereas cytosolic dropped to several percent in older animals. Interestingly, the contribution of the nuclear fraction did not change substantially with age. Next, we noticed that each type of peptidase activity contributed unevenly to this effect. ChT-L activity (Fig. 2c) followed the already described distribution pattern with prevailing activity residing in the microsomal fraction. In contrast, T-L activity (Fig. 2d) was almost evenly distributed between cytosolic and microsomal fractions (about 45% each) in young mice. Older animals showed a drastic drop down to 10% in contribution of the cytosolic fraction with the gain in the microsomal one. Finally, PGPH activity (Fig. 2e) showed little change in the activity distribution with a slow increase of contribution from the microsomal fraction and decrease of the cytosolic. Since the input from the nuclear fraction to the total activity was relatively low, age related changes were more difficult to assess. Nevertheless, we observed a relatively higher contribution of T-L activity in a nuclear fraction than in other fractions.

3.2 Specific activities of proteasome decline with age

We analyzed specific activities of proteasomes calculated by two ways: per total protein in the extract or per total amount of catalytic core particle. In the first case the data reflect contribution of proteasomal activities to the overall protein turnover in the subcellular compartments (protein load or protein challange). In the second case the data reflect the enzymatic state of proteasomes.

When the specific activity was calculated per total protein in the extract, each type of active center showed the highest specific activity in the microsomal fraction and the lowest in the nuclear fraction. None of the activity in these two fractions revealed statistically significant age dependent changes (Fig. 3). The microsomal fraction showed an age dependent decreasing trend in ChT-L and PGPH activities, whereas T-L activity remained unchanged. In contrast, ChT-L activity was unaffected in the nuclear fraction. Interestingly, in this fraction only a three-fold drop in T-L activity between 6 and 24 mos old mice was statistically significant. Additionally, a weak decreasing trend in PGPH activity was observed in the nuclear fraction. Surprisingly, all three specific activities showed statistically significant drop in the cytosolic fraction of older animals (Fig. 3). The six-months old animals always exhibited in this fraction up to 5-fold higher activities, when compared with older mice, although activities in 12 and 24 mos old animals remained similar. ChT-L decreased about 80% and 60% in 12 and 24 mos

old animals, respectively. The observed differences between 6 and 12 mos, and 6 and 24 mos were statistically significant (1 way ANOVA). More then five-fold decrease of T-L activity was noted between 6 and 12 or 24 mos old mice. Finally, PGPH activity dropped about four fold from the level determined in 6 mos to 12 and 24 mos animals.

Next, we examined changes in specific peptidolytic activity calculated per amount of the α7 subunit. Since the α7 subunit constitutes an obligatory and non-replaceable component of the catalytic core proteasome (20S) present in all the proteolytically active complexes, amount of this subunit was used to estimate the total proteasome content. To exclude a possibility that the free α7 subunit unincorporated into CP interferes with the estimation we separated proteins in fractions with native gel electrophoresis followed by the Western transfer and probing the membranes with specific antibodies. We found that the α7 responsive material traveling faster than CP constituted less than 3% of its total amount (not shown). Therefore, we concluded that the content of this subunit is suitable to estimate concentration of CP. We determined that also α4 and β4 subunits can be used for that purpose. The studies with β4 was performed with only 4 sets of animals, therefore the data were not included in the complete statistical analysis. Hence, any changes in specific activity that do not mirror the alterations in the content of α7 subunit may indicate differences in exchangeable subunit composition of CP and/or formation of distinct types of larger complexes. Interestingly, the content of α7 subunit did not change substantially with age in any of the fractions (Fig. 4). Its distribution between the fractions roughly followed that of total peptidolytic activity with the most of α7 subunit detected in the microsomal fraction (about 60%). The cytosolic α7 accounted for 30%, whereas nuclear for 10% of the total subunit content.

Total specific peptidase activity appeared more evenly spread between fractions when expressed per amount of the α7 subunit (that is, per amount of catalytic core proteasome times fraction volume) than expressed per amount of total protein (Fig. 5). In particular, the dominance of microsomal fraction activity was less striking. At the same time, the input from the nuclear fraction was more pronounced. Additionally, we observed even stronger an age dependent increase of microsomal fraction share in all three activities. Generally, all the described earlier patterns were detectable with the marked age related decline of all the activities in the cytosolic fraction when the specific activities were calculated per amount of the α7 subunit (Fig. 6, compare with Fig. 3).

3.3 Age dependent changes in specificity of proteasomes are pronounced in cytosolic and nuclear fractions

We also tested whether catalytic specificity of proteasome changes with age. As a measure of the specificity we used a ratio of ChT-L to T-L specific activity and a ratio of ChT-L to PGPH specific activity. Changes in these ratios may indicate alterations in amount of immuno and housekeeping proteasomes and shifts in prevailing types of larger complexes. Although T-L always constituted the predominant activity, we noticed that each fraction was characterized by a unique set of these ratios. The cytosolic fraction of young mice exhibited about a 4-fold excess of T-L over ChT-L activity and about a 2-fold excess of ChT-L over PGPH activity (Fig. 7a). In contrast, the microsomal fraction showed only two-fold excess of T-L over ChT-L activity and balanced ChT-L and PGPH activites. In turn, ther was lmost 6-fold excess of T-L versus ChT-L activity but similar levels of ChT-L and PGPH activities in nuclear fraction. The activity ratios in the microsomal fraction remained unchanged in older animals. In contrast, the cytosolic and nuclear fractions showed systematic age related changes leading to only two-fold excess of T-L over ChT-L activity in 24 mos old mice (Fig. 7b). However, the reason for this decline was different in these two fractions. In cytosolic fraction, both the activities decreased, although the T-L declined more profoundly. In contrast, in the nuclear fraction only the T-L activity dropped substantially, whereas ChT-L activity remained invariable.

3.4 Statistically significant age associated changes in protein level of selected proteasome subunits are limited to the cytosolic fraction

We tested protein expression levels of proteasome subunits with Western blotting in separated subcellular fractions of mice livers (Fig. 8, 9 and 10). We examined subunits representing 20S core particle, 19S regulatory particle, and 11S activator. Examples of the Western blots and corresponding standardized contents per mg of total fraction protein of selected subunits are shown in Fig. 8. Age related changes in individual protein levels followed a few distinct patterns. The majority showed either a systematic decreasing trend (Fig. 10) or a sharp drop between 6 and 12 mos animals and no change between 12 and 24 mos old mice (for example catalytic subunits). Occasionally, a decrease only in the oldest animals was observed, for example Rpn7 in the nuclear fraction (Fig. 10C). Rarely, a weak increasing trend was detected as in the case of nuclear β2 and LMP7 (Fig. 10C). Levels of many subunits did not follow any age related pattern. Surprisingly, we noticed that age related differences in protein levels were statistically significant for cytosolic β1 and PA28α subunits ($p < 0.05$, Fig. 9). The level of β1 subunit decreased by about 40% and 80% in 12 and 24 mos vs. young animals, respectively, however only the latter difference was statistically significant. In contrast, the differences in PA28α levels between both 6 and 12 and 6 and 24 mos old animals were statistically significant (Fig. 9).

The level of α4 and α7 subunits decreased slightly in the nuclear fraction but it did not change in cytosol and microsomal fractions (Fig. 10) suggesting the decrease of the total amount of proteasome only in the nuclear fraction. It was expected that the decline in a level of housekeeping catalytic subunits would be compensated by the increase of corresponding immuno subunits where the fixed levels of alpha subunits were observed. However, a level of LMP2 (β1i) that substitutes β1 subunit in immuno proteasome did not meet this expectation. Similarly, a drop in a level of β5 subunit in a microsomal fraction was not accompanied by an increase of LMP7 (β5i) level. To the contrary, we noticed a decreasing trend of MECL1 (β2i) in all fractions, LMP7 in microsomal fraction, and LMP2 in nuclear fraction (Fig. 10). Interestingly, a possible compensation was observed in the nuclear fractions within β5/LMP7 and β2/MECL1 pairs, however the decreasing trend was exhibited simultaneously by β5 and MECL1 subunits complicating formation of the classical immuno proteasome containing exclusively the catalytic immuno subunits.

Only three subunits of the 19S regulatory particle showed systematic changes associated with age (Fig. 10). Although a level of Rpn2 decreased in each fraction, only in the nuclear fraction of the oldest animals the drop was abrupt. The substantial decrease of Rpn7 level was observed in microsomal and nuclear fractions. Again, in the latter fraction the drop was sharp. Finally, the level of Rpn10 decreased in cytosolic and microsomal fractions. α and β subunits forming one type of PA28 activator complex showed a clear decreasing trend in cytosolic and microsomal fractions, and in the former the difference was statistically significant (Fig. 9). Additionally, the β subunit of PA28 also displayed a similar decreasing trend in nuclear fraction. In contrast, the γ subunit, a component of homo hexameric type of PA28 complex did not reveal age associated variation in any of the fractions (Fig. 10).

3.5 Cluster analysis reveals the most pronounced age related trends in activities and subunit contents in nuclear fraction

We attempted to subject the protein expression levels and peptidases activity data to cluster analysis to determine whether they assemble into common patterns of age associated changes (Fig. 10). Cluster analysis was performed on data separately standardized within each fraction. A presence of three distinct clusters was detected in the cytosolic fraction (Fig. 10A). The first cluster characterized by a systematic decrease consisted of peptidase activities, β1 and MECL1 catalytic subunits and PA28α. Remaining catalytic subunits, α subunits, Rpn2, and PA28β were

present at fairly steady high levels whereas other Rpn subunits and PA28γ stayed at the constantly low level creating the second and third cluster, respectively. Collectively, young animals formed very distinct “high level” group in this fraction, whereas other two age groups were relatively similar to each other. Clusters were less clearly formed in the microsomal fraction (Fig. 10B). Although the declining trend could also be noticed within PA28α, β subunits and peptidolytic activities but the majority of catalytic and 19S subunits indicated a weak increasing trend or could not be classified to any systematic pattern. Similarly to cytosolic fraction, the 6 mos old animals created a separate group. However, in this case it was much less pronounced. In contrast, the nuclear fraction revealed a member - rich cluster showing a strong declining trend (Fig. 10C). Interestingly, catalytic subunits and PA28α were placed outside this cluster since instead they followed a weak increasing trend. The cases assembled in the category of 24 mos old mice showed a prominently unique pattern characterized by low levels of majority of subunits. Such arrangement was in striking contrast with other fractions where the young animals showed a distinctly separate pattern (Fig. 9, 10).

4. Discussion

4.1 Catalytic load of proteasome changes with age in a fraction-specific manner

4.1.1 The cumulative proteasome activity decreases with age—It is well established that protein catabolism is diminished as an organism ages. Catalytic activites of proteasome involved in degradation of bulk of intracellular proteins follow this trend. Significant decrease in the activity has been recorded for bovine eye lens, rat liver, kidney, lung, muscle and heart, human skin, epidermal cells, fibroblasts and lymphoblasts, brain of rat, mice and marmosets, and many other organisms (reviewed by Vernace et al., 2007a; Gaczynska et al., 2001). Our data strongly support the previous observations but also add new clues to sources of the decline. We carried the peptidase activities measurements without the detergent (SDS) activation. Therefore, we assume that our preparations contained a mixture of different complexes closely resembling their distribution *in vivo*, and that our activity data reflect that *in vivo*-like distribution. The cumulative peptidase activity decreased by about 40% in 12 mos old mouse liver what was in line with the 50% decline of protein degradation found in rat livers (Shibatani et al., 1996). A similar decline in rat livers was just reported by Dasuri et al. (2009) and Breusing et al (2009). Unexpectedly, the specific activity of proteasomes measured in 24 mos mouse liver did not further decrease. The 12 mos old mice are fully mature so it is unlikely that the observed drop could be explained with an ongoing maturation process. Many studies have been limited to comparison of two age groups of animals. Therefore, broader assessment of the dynamics of changes is difficult. Additionally, the analysis is often complicated by comparison of organs harvested from animals that chronologically represent young and middle-aged rather than old groups. Nevertheless, a profound drop of protein turnover (Birchenall-Sparks et al., 1985; Lewis et al., 1985), RNA synthesis (Lewis et al., 1985) and mitotic activity of hepatocytes (Schapiro et al., 1982) was detected even in the middle-aged group of rats. Interestingly, these changes associate with decline of body mass growth and the maximum of fertility, conforming the notion of aging-specific changes in middle-aged animals (Miller and Riegle, 1980). Accordingly, Hayashi and Goto (1998) collecting data over expanded number of time points found a linear decline of protein turnover and a decrease in all the proteasomal peptidase activities with age. In contrast, recent data collected over several time points support the uneven decrease of ChT-L activity (Breusing at al., 2009).

4.1.2 Proteasome activity involved in protein quality control resists the age-related changes, whereas activity involved in nuclear signaling declines most profoundly—Another intriguing observation was a high association of all the peptidolytic activities with the microsomal fraction that even further increased with age, apparently at the expense of cytosolic proteasome. Immunogold electron microscopy studies on rat hepatocytes

found about 14% of proteasomes associated with the ER (Rivett et al., 1992). Analysis of subcellular fractions from rat liver pointed at smooth ER and cis-Golgi as the microsomal localization (Palmer et al., 1996). Our tests assured that there were virtually no cross contaminations of the separated fractions. However, proteasome may associate with membranous elements other than ER and Golgi apparatus (Pal et al., 1988), and with membrane-bound cytoskeleton (Ryabova et al., 1994; Arcangeletti et al., 1997). Therefore, we suspect that an enrichment of the microsomal fraction with other membranes resulted in the high content of proteasomes. Another culprit may be activation of CP by membrane components (Arizti et al., 1993). its posttranslational modifications or a presence of CP distinctively decorated with activator modules (Hanna and Finley, 2007). We plan to perform electrophoretic separation of the complexes under native conditions to shed light on the issue (Elsasser et al., 1995)

To best of our knowledge this is the first study where in the context of aging the three peptidolytic activities and their intracellular distribution were studied in parallel (Fig. 9). All the individual activities followed the general distribution pattern already described for the cumulative activities. This pattern was illustrated most sharply by more than two-fold drop of the T-L activity share in cytosolic fraction when older mice were compared to 6 mos animals, thus leading to the substantial input increase of the microsomal fraction. Such a shift also shown by other peptidase activities suggests an enhanced role of proteasomes associated with this fraction as aging progresses. Importantly, microsomal fraction contains proteasomes involved in ERAD (ER associated degradation) that constitutes protein quality control machinery (McCracken and Brodsky, 2005). It is possible that age-related increase in load of the unfolded or misfolded translation products and damaged proteins handled by ERAD induces the observed increase in contribution of microsomal proteasomes. At the same time the general metabolic activity in cytosol and proliferative potential of a nucleus decline. The redistribution of intracellular 20S particles is possible only when a nuclear envelope disintegrates during mitosis (Wojcik and DeMartino, 2003). In all other stages of cell cycle the proteasome is unidirectionally transported to nucleus, however the system responsible for that process is less efficient with age. Therefore, we suspect that more proteasomes are retained in ER of older mice. Since only activities of the cytosolic proteasome declined systematically with age, it seems that the enzyme from this compartment can be linked to a partial impairment of the general housekeeping degradation of the bulk of cytosolic proteins.

We determined an activity of each individual active center with short model substrates to avoid the specificity bias associated with longer polypeptides. Since ChT-L activity breaks peptide bonds after hydrophobic residues that are common in majority of proteins, it is considered the “workhorse” specificity of proteasome generating relatively short products. Therefore, a robust ChT-L activity is predominantly associated with the efficient destruction of dysfunctional proteins. Then, subtle shifts in partition of the other two specificities would indicate their targeted role in the regulated formation of specific products (Kisselev et al., 2006). Indeed, proteasomes from each fraction demonstrated the unique peptidolytic specificity expressed as a ratio of ChT to T-L and ChT to PGPH activities. Strikingly, microsomal and nuclear fractions showed opposite patterns of specificity. Expectedly, proteasomes from microsomal fraction, involved in quality control, were more active toward the hydrophobic substrates. The nuclear proteasomes were uniquely equipped to destroy basic substrates. The ratio of ChT-L to T-L activity increased with age both in cytosolic and nuclear fractions. The faster decrease of T-L activity of cytosolic proteasomes than ChT-L indicates that likely the housekeeping functions of proteasome were particularly relevant in liver physiology as the organ aged. In turn, the nuclear proteasomes forfeited their T-L activity whereas their ChT-L activity, although low, remained relatively constant. Such a shift in specificity might follow the age dependent decrease of proliferative and transcriptional activity of liver cells.

4.2 The spatial and functional organization of proteasome assemblies changes in aging liver

4.2.1 Changes in specific activities of proteasome are compartment and age dependent although they are not accompanied by changes in the total content of CP—Since alpha subunits are constitutive elements of every CP, it is believed that alterations of their level reflect the changes in amount of all proteasomal complexes. Indeed, we detected only minuscule amounts of the free α4, α7 and β4 subunits not incorporated into the complexes. In agreement with numerous previous data, the level of α4 and α7 subunits generally did not change with age (reviewed by Vernace 2007b, Gaczynska et al., 2001). Only the nuclear alpha subunits showed a weak and not statistically significant decreasing trend. Interestingly, recalculation of peptidolytic activity per amount of alpha subunits did not influence the previously described trends. However, the contribution of the specific activity of nuclear proteasome to the total peptidolytic activity was much higher than those calculated per total amount of fraction specific protein. This observation mirrored higher specific activity of nuclear proteasome resulting from several times lower concentration of alpha subunits in nuclear than in cytosolic and microsomal fractions. Therefore, the more prominent specific activity share of the nuclear proteasome took place mostly at the expense of the microsomal fraction, where high total activity was accompanied by high content of CP.

4.2.2 The compromised cytosolic activity of proteasome can be partially explained by the decline of PA28α content—The constant level of alpha subunits during aging suggest that other factors than the total amount of 20S proteasome influenced the observed decline in the peptidase activities. Several factors should be considered: (1) content of immunoproteasome relative to housekeeping CP; (2) content of RP, activators and PI31; (3) posttranslational modifications (PTM) of the proteasome; (4) oxidative or other deleterious modifications. Western blotting showed that with age content of many subunits either declined or did not follow any clear trend. β1 subunit and PA28α, the component of 11S activator, were the only two subunits, which decline with age was statistically significant in the cytosolic fraction. The subunit β1 is responsible in the housekeeping CP for the PGPH activity and its changes followed that of the corresponding peptidolytic activity. We expected that the absence of the 1 subunit would be compensated by an increased content of matching β1i/LMP2, however we could not detect such a tendency. The imbalance in content of the catalytic subunits may be explained by formation of hybrid proteasomes containing in a single complex both elements of housekeeping and immunoproteasome (Klare et al., 2007; Dahlmann et al., 2000). An input from a small pool of free CP subunits is worth exploration, however is less plausible due to a quick degradation of such unincorporated subunits under the normal physiological conditions. Likely, CP subunits do not follow the example of PA28γ (Gao et al., 2004) and Rpn10, constitutively found in a free “reserve” pool (Matiuhin et al., 2008).

The PA28α forms a hetero- hexameric complex with PA28β subunit that activates all three peptidase activities of 20S proteasome. Based on comparison of mRNA quantities (El-Khodor et al., 2001), the amount of the 11S complex is limited by supply of the PA28β. Although the decline of PA28β was less impressive than PA28α, it may result in a lower availability of the 11S activator. Such the decrease may further explain the lower ChT-L and T-L activities observed in older fractions.

4.3 Cluster analysis confirms general age related decline in catalytic potential of the proteasome and points at the possible mechanisms

Although the majority of changes in subunit levels did not meet conditions of the stringent statistical significance test, we decided to explore if they would form a pattern indicating coordinated alterations of a particular type of complexes. To reach this goal, we assembled all the data into groups using cluster analysis. Classification of the components in the cytosolic fraction confirmed the overall activity decline supported by the decrease of the catalytic

subunits and decrease in 11S activator level. The lower amount of 11S-activated proteasome may lead to release of shorter products triggering a significant shift in degradation specificity in older animals. A decrease in the housekeeping performance of proteasome would be expected, especially since components of 19S cap also showed the declining trend with age or constant low levels. In contrast, immuno subunits were classified as proteins expressed at constantly high levels. Since immunoproteasomes are often decorated with PA28αβ, the presented activator immunoproteasome imbalance further points at the major refocusing of the catalytic specificity of cytosolic proteasome in aged tissue. Interestingly, Dasuri at al. (2009) found in aged rat liver homogenates an increased level of PA28α. The discrepancy may arise from the fact that this group measured a level of PA28 forming a complex with proteasome and we tested a level of the total available PA28. The observation may reflect age dependent changes in stability of proteasomal complexes.

Although the cluster analysis of the microsomal fraction components also showed decline in activity, it was mostly limited to Ch-T and T-L peptidases and it was not supported by decrease of catalytic subunits. Similarly to the cytosolic counterpart, the microsomal PA28αβ activator also showed the strong decrease. Surprisingly, several components in this fraction revealed a weak increasing trend including PGPH activity, immuno catalytic subunits and activator PA28γ. Such changes indicate a presence of less active proteasomes with the substantially altered specificity leading to more effective digest of acidic substrates, the content of which may increase with age in this fraction (Gavilan, et al., 2009).

The majority of nuclear components, with exception of PA28α and β2 subunits, demonstrated a clear declining trend (Fig. 10C). Such drastic changes may indicate a shutdown of proteasomal functions in the nucleus resulting from the decreased metabolic activity of this organelle followed by specific removal of its protein components. It is interesting to what extent the lower levels of 19S components influence the degradation of ubiquitinated substrates versus postulated non-proteolytic, transcription-related roles of the free RP. Likely, the regulatory functions of 19S cap are diminished in older mice.

Taken together, the collected data emphasize the need to individually study each subcellular fraction since they demonstrate unique patterns of age related changes (Fig. 11). The activity decline and alterations in the specificity are the most characteristic features of the aging cytosolic fraction. That can be at least partially explained by the lower supply of PA28αβ activator and β1 subunit. Such status is likely affecting proteolysis of short lived and damaged proteins. In the declining proteolytic environment the bulk of duties are shifted toward microsomal associated proteasomes. Although these proteasomes have to perform with a lower amount of PA28αβ activator, their total activity and specificity remains steady with age. The most intriguing in this context is the status of nuclear proteasomes. Quantitatively their contribution to the total intracellular activity seems minuscule, however a strikingly different assemblies, both in terms of activity, specificity and subunit composition, are directed to this compartment dependent on the age of animal. We speculate that the changes may affect quantity and quality of transcription factors and proteins involved in chromatin remodeling, in addition to putative non-proteolytic 19S RP dependent events. Interestingly, the cluster analysis of association between age groups shows that the nuclear proteasomes isolated from the 24 mos old animals stand out as a case with the clearly decreased content of the majority of variables. Other fractions demonstrate closer similarity between proteasomes from 12 and 24 mos old animals leading to a separate class left for “young” proteasomes. In that respect the properties of nuclear proteasome seems to constitute the best marker of aging. Likely, the peptidase specificity of cytosolic proteasomes represents another valuable marker (Fig. 11).

In conclusion, during aging, proteasomes lose the catalytic potential what is in part controlled by the decline of PA28 activator and 19S RP levels in a manner unique for each subcellular

compartment (Fig. 11). The observed structural and functional differences are only a part of age-related changes in other components of the UPP and in protein quality control. It remains to be established whether the described changes in livers represent a common profile shared by other organs. We suspect that the organs exhibiting a similar degree of the terminal differentiation may follow a very similar pattern of age-associated alterations that can be modified only to a limited extent by the specialized functions of the organ.

Acknowledgments

The work was supported by NIA grants T32 AG021890 and P30 AG13319, as well as R36 AG033401 (KR) and R03 AG021267 (MG). We also thank the Sam and Ann Barshop Institute for Longevity and Aging Studies, and especially Dr. Arlan Richardson and Dr. Walter Ward, for continuous support and inspiration.

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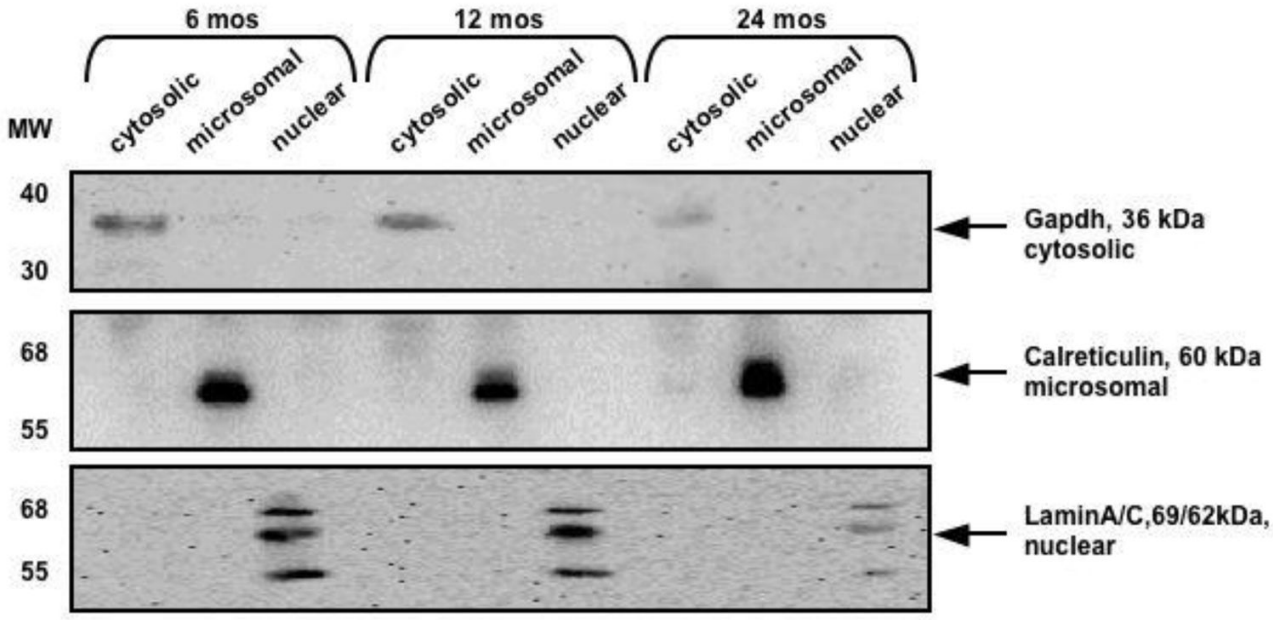

Fig. 1.

The liver lysates were successfully separated into cytosolic, microsomal and nuclear fractions, as demonstrated by Western blotting with antibodies detecting marker proteins exclusively present in the three subcellular compartments. Approximate positions of molecular weight (MW) standards in kDa are indicated.

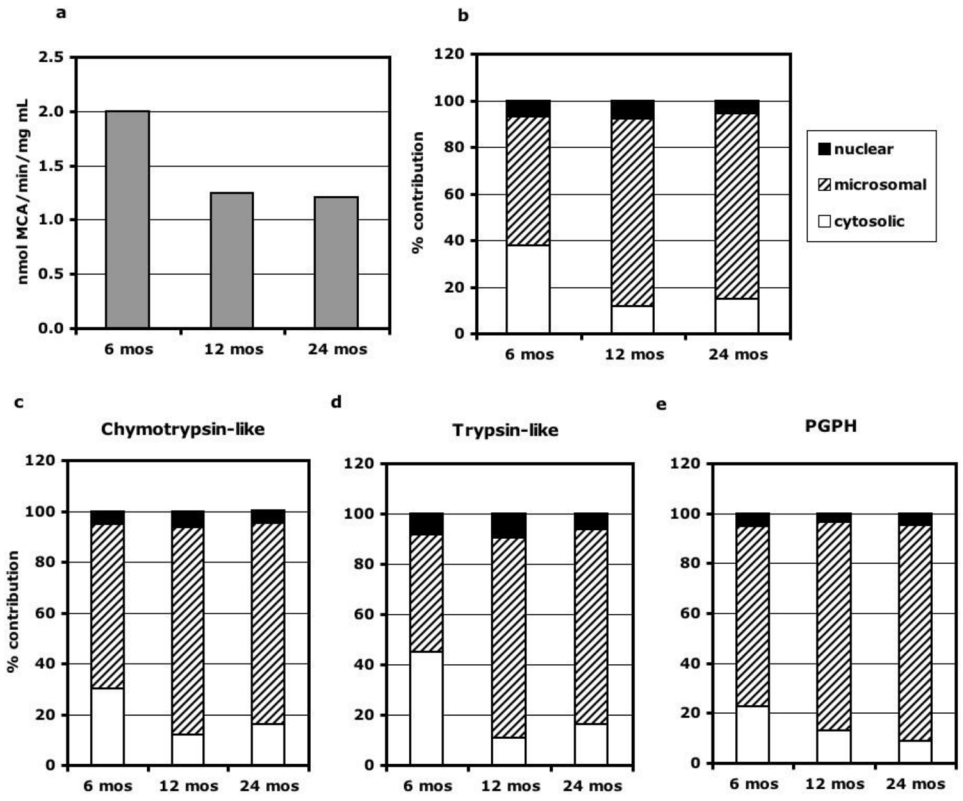

Fig. 2.

Total specific peptidolytic activity of proteasome declines with age (a) whereas contribution of each fractions to the total peptidolytic activity (b) and contribution to the individual peptidase activities (c–e) is strikingly distinct. The activity was calculated as a sum of total specific activities (specific activity multiplied by fraction volume) of all peptidolytic sites (ChT-L+T-L+PGPH) in all the fractions (cytosolic+microsomal+nuclear). In panels (b) through (e) the total activity for each age group was normalized to 100%.

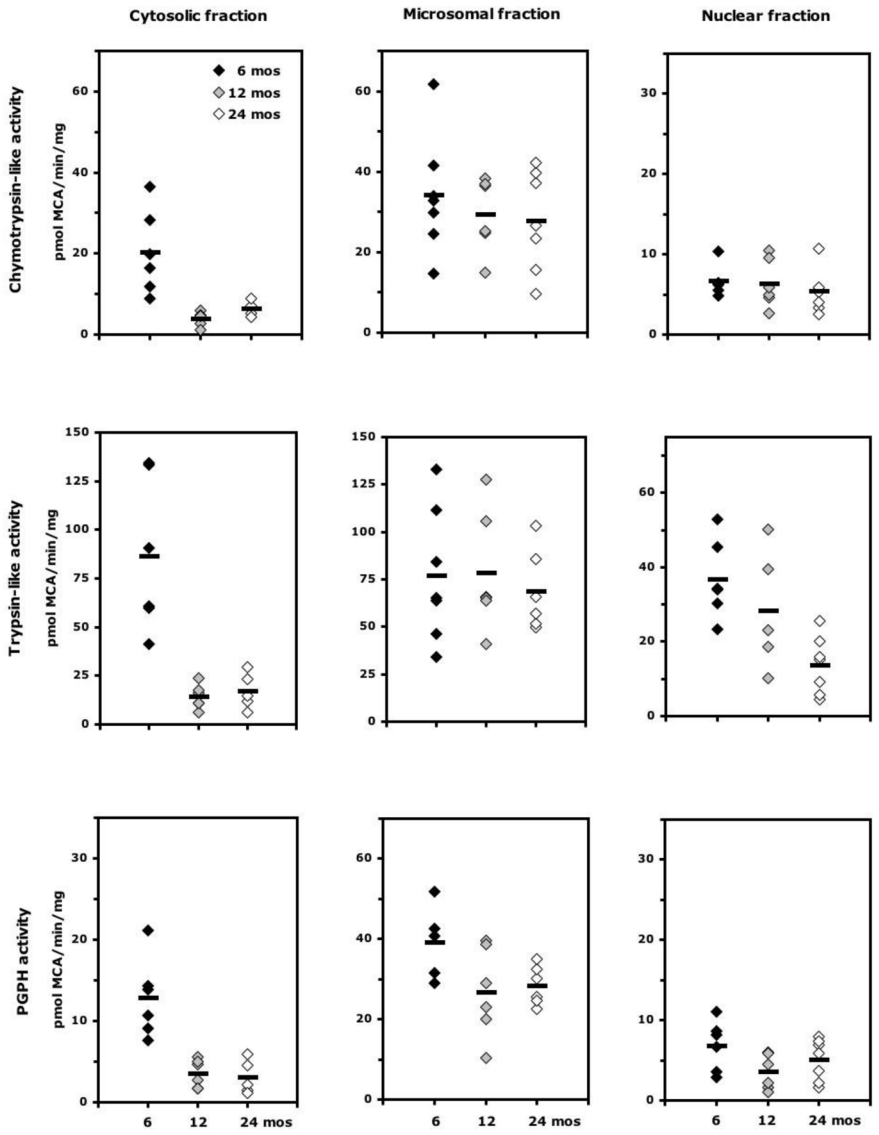

Fig. 3.

Specific peptidase activities showed the statistically significant decline only in the cytosolic fraction when older mice were compared to 6 mos old animals. The microsomes associated proteasomes exhibited no clear change in peptidase activities. Nuclear proteasomes revealed weak declining trends. Although not statistically significant, the strongest declining trend in this fraction was demonstrated by T-L activity. Specific activity was calculated as a reaction rate per amount of total protein in the corresponding fraction what approximates a total catalytic challenge potentially faced by proteasome.

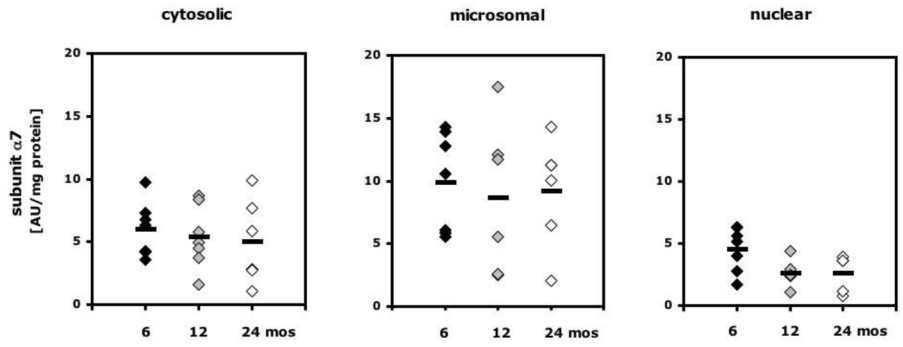

Fig. 4.

Western blot detected levels of the α7 subunit do not change with age in any of the fractions indicating steady levels of 20S proteasome. The partition of α7 subunit pool between the fractions was identical in all the age groups (approximately 28% in cytosolic, 61% in microsomal and 11% in nuclear fraction).

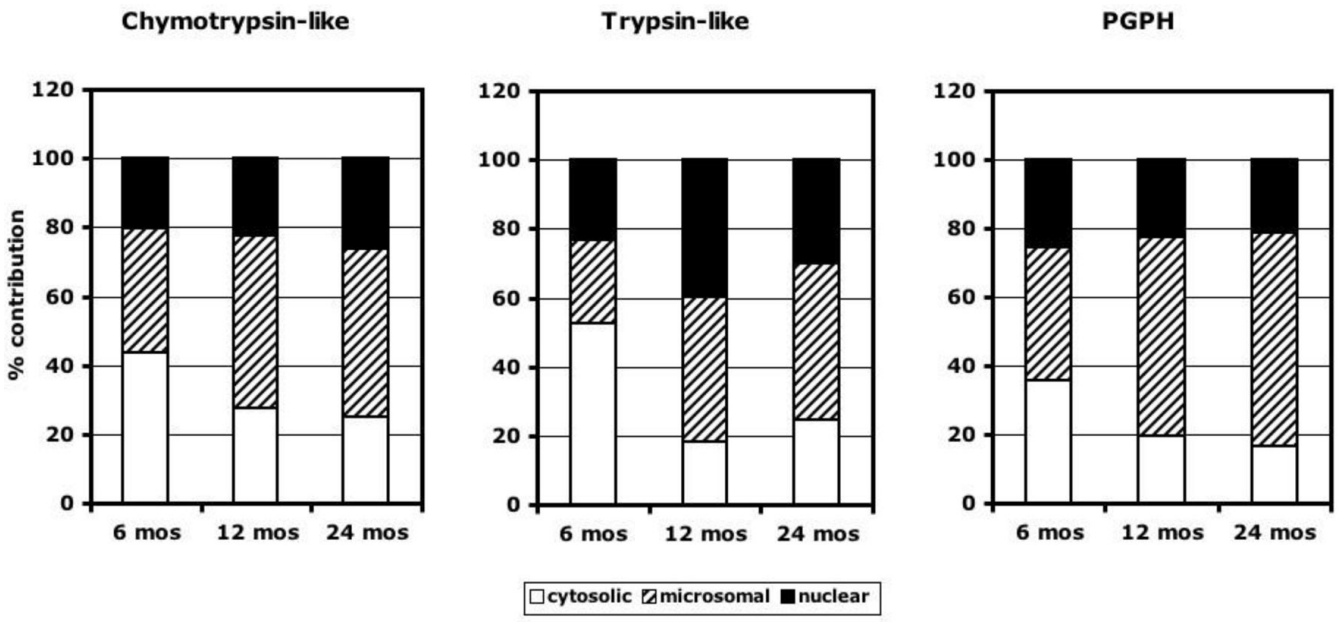

Fig. 5.

Partition of specific activities between individual fractions reveals age dependent changes. Specific activity of proteasome was recalculated per amount of the α7 subunit to approximate peptidolytic capacity of the proteasome. In comparison with Fig. 2c-e a contribution of proteasomes from the microsomal fraction is less pronounced whereas two other fractions show a marked increase of their respective contributions. The difference between the contributions of specific activities calculated in two distinct ways is especially striking in the case of T-L activity.

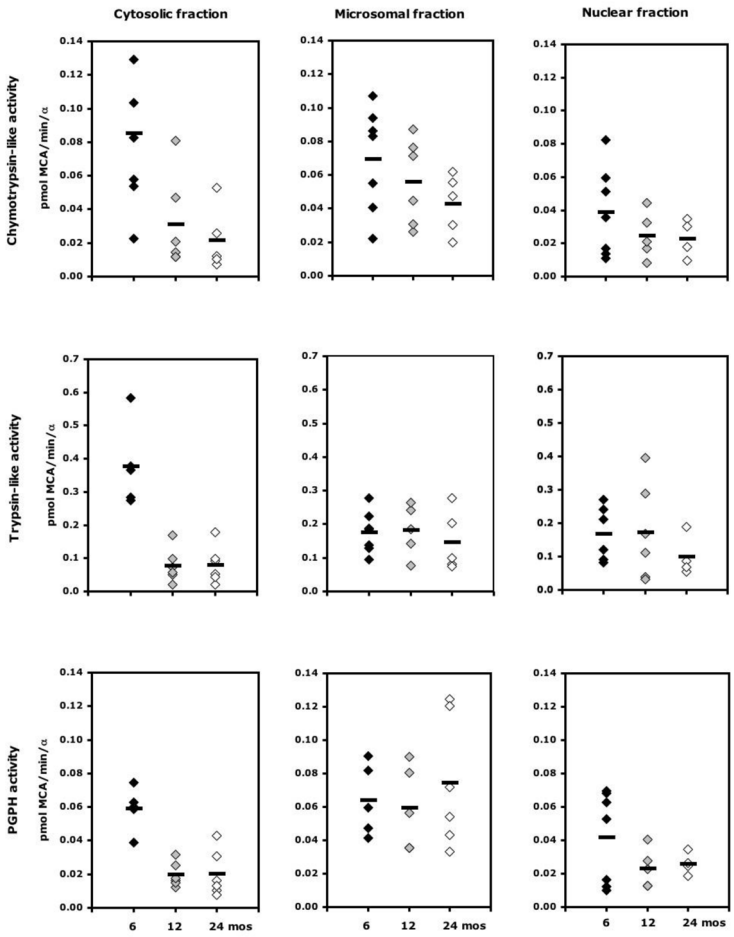

Fig. 6.

Specific activity of each peptidase calculated as a reaction rate per amount of the α7 subunit closely follows the pattern of changes observed when calculations were performed per amount of total protein in the fraction (compare with Fig. 2). Since the variance is expectedly higher here, only differences in T-L and PGPH specific activities in cytosolic proteasomes still are statistically significant when older animals are compared to 6 mos old mice.

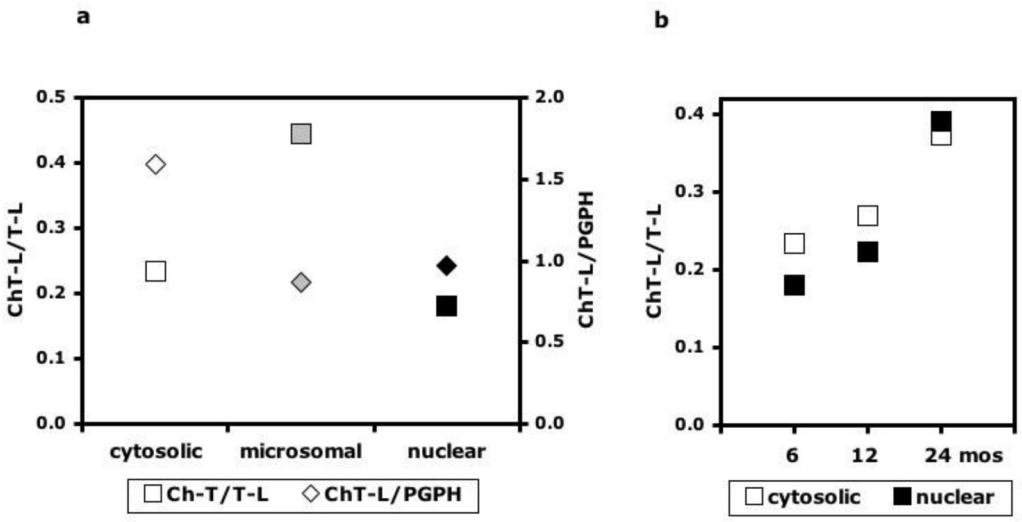

Fig. 7.

Proteasomes from the individual subcellular fraction are characterized by distinctive peptidase specificity expressed as ratio of the ChT-L to T-L and ChT-L to PGPH activity (a). There is an age associated increasing trend of the ChT-L to T-L activity ratio in cytosolic and nuclear fractions (b). The specificity of proteasome can be approximated by ratios between specific activities of the major proteasomal peptidase. Changes in the specificity likely result in shifts of the cleavage rates of distinct substrates and a pattern of generated products.

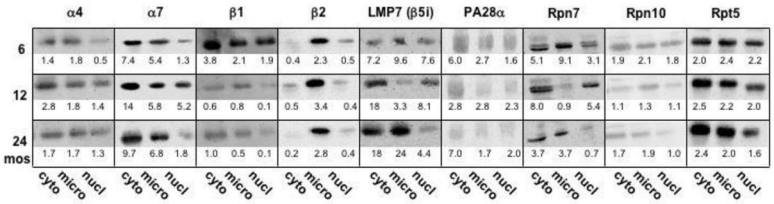

Fig. 8.

Western blot detected contents of proteasomal subunits depend on subcellular fraction and age of the liver tissue. Representative Western blots probed with specific antibodies against (from the left): constitutive subunits of CP (α4, α7), catalytic subunits of CP (β1, β2, β5i), α subunit of 11S activator and subunits of RP (lid: Rpn1, hinge: Rpn10, base: Rpt5) are shown. The numbers represent contents of subunits expressed in arbitrary units per microgram of protein in the extracts, standardized against the known amount of protein A conjugates loaded on each gel.

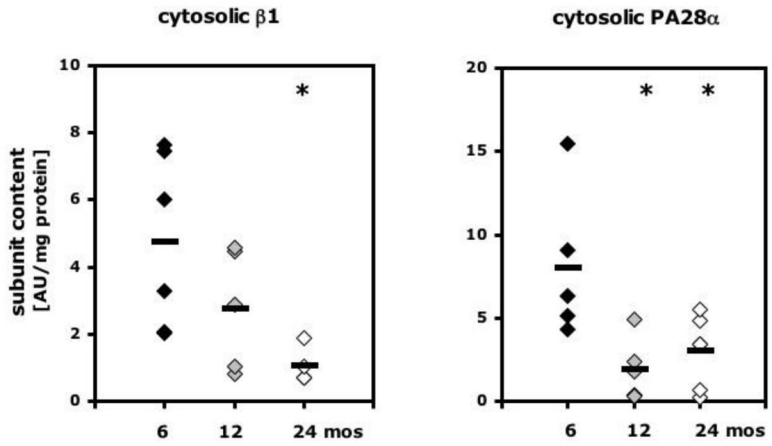

Fig. 9.

Western blot detected levels of the cytosolic subunit β1 and α subunit of PA28 activator decline with age. The differences are statistically significant between 6 and 24 mos old animals for β1 and between 6 and 12 and 6 and 24 mos old mice for PA28α at $p < 0.05$.

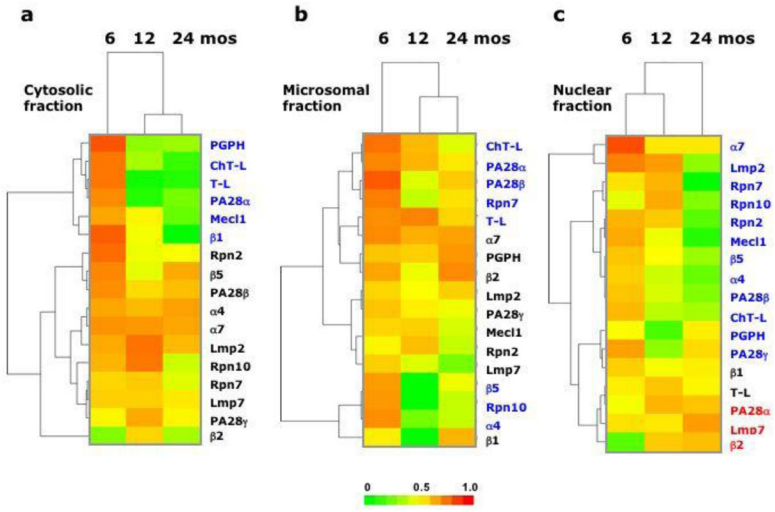

Fig. 10.

A heat map showing results of the cluster analysis of variables including normalized peptidolytic activities and quantities of Western blot detected subunits of proteasome (shown in rows). Age groups organized in columns represent cases. Normalization was performed separately for each fraction. Results obtained with Cluster 3.0 analysis are shown, however other software packages produced virtually identical association of variables. Heat maps were prepared with TreeView v. 2.11. Color scheme corresponds to the normalized values of variables where dark green represent the lowest (close to 0) and red the highest (close to 1) values. The lengths of tree branches are proportional to a relative similarity between variables and between cases. Blue or red color of the font used for individual variable description denotes a clear declining or rising trend, respectively.

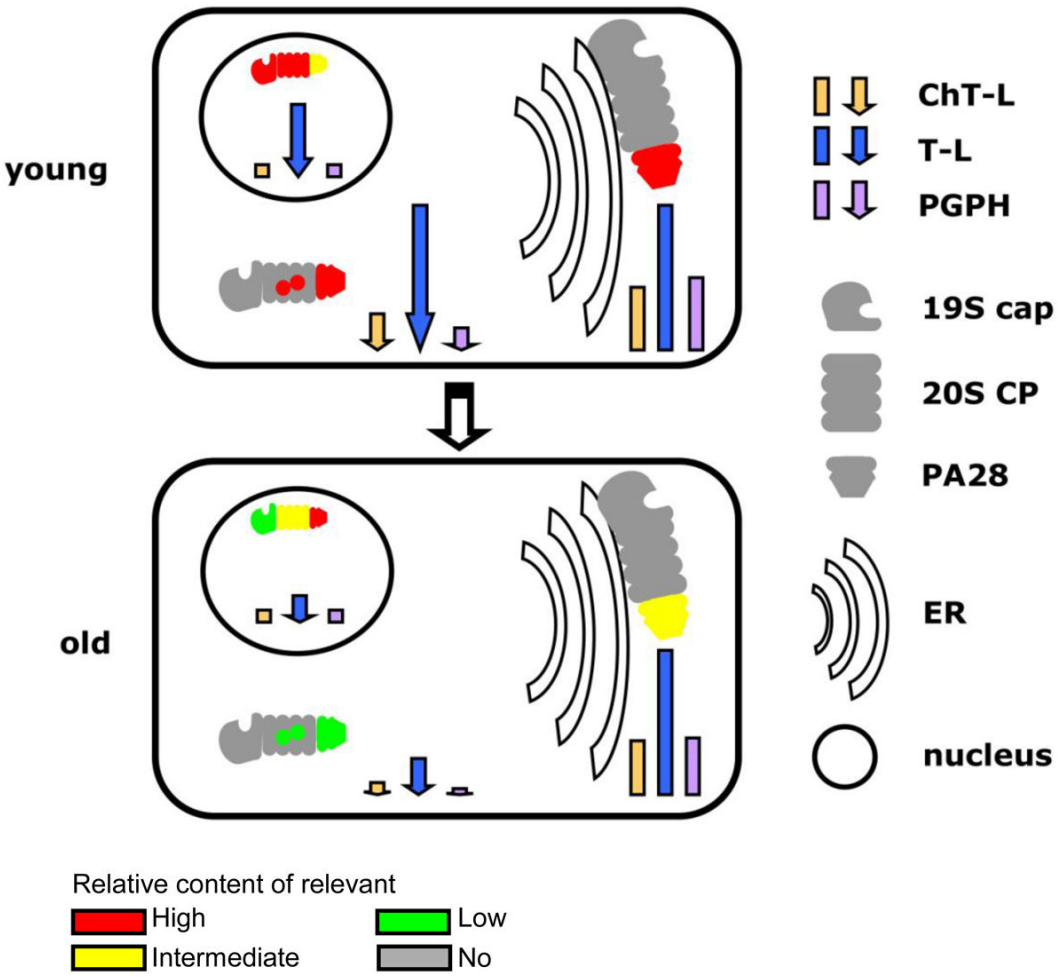

Fig. 11.

A model summarizing age related changes in proteasome distribution, subunit content, and peptidolytic activity. For simplicity only two age points are shown: “young” cell corresponds to 6 mos old mice whereas “old” cell represents collectively 12 and 24 mos old animals. The cells are divided into three sectors representing cytosolic, microsomal (block arcs), and nuclear (a circle) fraction. Changes in peptidase activity are denoted either by a rectangle if no trend was detected or a downward pointing arrow if a decreasing trend was found. The heights of rectangles and arrows are proportional to specific activity they illustrate. The areas of 20S CP silhouette are proportional to their partition between the fractions as determined with a level of the α7 subunit. Areas of 19S cap and PA28 are not proportional to levels of these complexes and for simplicity follow the area of 20S CP. Generalized changes between the age groups in the relative content of subunits forming complexes are coded with color of the silhouettes: red – high, yellow – intermediate, green - low level. Grey color indicates that no change was found. Two small colored dots in the β ring of 20S CP represent β1 subunit.