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Proteomic analysis of the amyloid precursor protein fragment

C99: expression in yeast

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Abstract

The accumulation and aggregation of fragments of amyloid precursor protein (APP) are central to the development of Alzheimer's disease. The production of the small fragment C99 is thought to form the rate-limiting step in the APP processing pathway, which can lead to the production of the toxic A β peptide. It has also been suggested that the proteasome contributes to APP catabolism. While the identities and aggregation propensities of many APP fragments have been studied *in vitro*, the sequences, structures, and cellular sources of fragments generated *in vivo* remains poorly elucidated. To better identify the specific APP fragments generated *in vivo* and to elucidate the role of the proteasome in APP processing, we developed a C99 yeast expression system. Using Zip Tip immunocapture, a specific anti-A β antiserum (6E10), and matrix-assisted laser desorption/ionization-time of flight mass spectrometry, we identified over one dozen APP-generated peptide fragments in wild-type yeast (*PRE1PRE2*) and over three dozen unique fragments in proteasome mutant cells (*pre1-1pre2-1*) expressing C99. Based on the identities of the immunocaptured species, we propose that defects in proteasome function are compensated by other proteases and that the combination of techniques described here will be invaluable to further delineate the APP processing pathway *in vivo*.

Keywords

Zip Tips; MALDI-TOF; Yeast; A β ; C99; Proteasome

C99 is a membrane-bound peptide generated from the amyloid precursor protein (APP)¹ by β -secretase cleavage. The production of this C99 fragment from APP is believed to be the rate-limiting step in releasing smaller aggregation-prone peptide fragments, such as A β peptide, from the membrane [1]. The accumulation of these APP fragments to critical concentrations is thought to ultimately lead to the neurodegeneration that is evident in Alzheimer's disease (AD) [2].

The structure and aggregation propensity of many APP fragments have been well characterized *in vitro* [3–7] though the generation of all of these fragments *in vivo* under physiological conditions has not been elucidated. This project aimed to address this shortfall. Also, by engineering mammalian cell culture systems some of the intracellular processing pathways for

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¹Abbreviations used: APP, amyloid precursor protein; AD, Alzheimer's disease; NEP, neprilysin; NEPLP, neprilysin-like protease; BSA, bovine serum albumin; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; CSF, cerebrospinal fluid.

APP have been identified [8–13]. However, given the use of pharmacological agents in most of these studies, the role of side effects from the agents used cannot be excluded. Nevertheless, these studies have provided useful information on the diverse spectrum of cellular proteases that contribute to the generation and/or degradation of APP and C99, including α -secretase, the γ -secretase protein complex, neprilysin (NEP; neutral endopeptidase), the neprilysin-like proteases (NEPLP), and insulysin [1,14–22]. The γ -secretase protein complex is an aspartyl membrane protease whose activity results in the production and release of the A β peptide [23]. In contrast, the α -secretase releases a luminal fragment of APP, which can prevent the formation of the toxic A β peptide [16,24]. NEP, NEPLP, and insulysin (which degrades proteins in a manner similar to that of NEP) [19,25,26] are also membrane-bound enzymes but they preferentially cleave the N-terminal peptide bonds of bulky hydrophobic residues into small (<40 to 60-residue) peptides [27]. Since NEP and insulysin have been shown to catalyze A β degradation *in vivo* [14,15,19,27–30], identifying fragments that result from these enzymatic activities is important in modeling the mechanisms of AD [30]. However, the complete panel of *in vivo*-generated APP fragments and their propensities to generate toxic, aggregation-prone species, remains ill defined. In addition, while the contributions of some fragments to disease pathology are understood, the physiological relevance of many fragments remains to be elucidated.

Another protease that may contribute to APP catabolism in mammalian cells is the proteasome [16,31]. The proteasome is a large (26S), multicatalytic cytoplasmic/nuclear protease known to process aggregation-prone secreted proteins that may otherwise lead to neurodegeneration [32]. Since proteasomal activity decreases with age and age is the biggest risk factor in the development of neurodegenerative diseases [22], it is not surprising that this abundant enzyme has been implicated in AD.

Although there are clear advantages to using mammalian cell systems to explore the cellular processing pathways for APP [8–13], the use of the yeast system can and has allowed for rapid initial pilot studies for many proteins linked to human disease [33]. Hypotheses developed using the yeast system can then be tested in mammalian cells. Because the secretory pathways and protein quality control machineries are conserved between yeast and mammals, because specific and/or rapidly acting temperature-sensitive yeast mutants exist that allow for the inactivation of specific cellular component(s), and because yeast do not express APP or C99 but can be engineered to synthesize these proteins [34,35], they are an excellent system for these studies. Yeast have also been engineered to express the γ -secretase complex [23] and were found to process APP (and its fragments) using endogenous α -[36–39] and β -[40] secretase activities that are similar to those identified in human cells. Yeast have also been shown to package APP into secretory vesicles in the same manner as seen in human cells [41].

In this study, we generated a C99 yeast expression system to directly examine the role of the proteasome in the processing of C99. The vector system chosen was one that has been shown to successfully express and secrete full-length APP from *Saccharomyces cerevisiae*. The gene is under the control of a galactose-regulated promotor and the protein contains an N-terminal “pre pro” sequence for the yeast α mating factor, which directs the protein into the secretory pathway (Fig. 1). This pre pro region is cleaved and removed by Kex2 in the Golgi [34]. We have now reengineered this vector system for the expression of C99 and have combined this system, with proteomic technologies, to directly investigate the role of the proteasome in the processing of C99. Because our system does not require the long-term addition of pharmacological agents, nonspecific side effects are minimized. Specifically, we have investigated how yeast compensate for compromised proteasome activity with respect to C99 processing. The results of our study support a role for the proteasome in C99 catabolism, provide new insights into the fragments generated *in vivo* in the presence of all existing cellular

proteases, and provide information on the important and to date overlooked role played by compensatory processing pathways that occur when proteasome activity is ablated.

Materials and methods

Yeast strains and manipulations

Cells were grown on standard medium using common handling procedures [42]. Wild-type (WCG; *MATahis3-11,15leu2-3ura3PRE1PRE2*) yeast strains or those deficient for proteasomal activity (WCG/11-21; *MATahis3-11,15leu2-3ura3pre1-1pre2-1*) [43] were transformed using the lithium acetate procedure essentially as described [44]. Cells were plated onto Synthetic Complete (SC) medium lacking uracil (SC–ura) that contained 2% glucose. The transformed yeast cells were incubated at 26 °C for 5 days and a resultant colony was streaked onto fresh SC–ura +2% glucose agar plates so that single colonies could be grown and isolated.

Construction of the C99 expression vector

C99 was expressed from JJB20 (a kind gift from Dr. R. Fuller, University of Michigan [34]). This multicopy (2- μ) plasmid is derived from YCp50 which contains pBM258, which is an *EcoRI–BamHI* fragment of the *GAL1–GAL10* intergenic region. The JJB20 construct also has MF α 1-100 engineered into it, which codes for the pre pro sequence of the yeast α factor mating pheromone. This is downstream of the pBM258 region. To insert C99 into the vector, PCR was used to generate *XbaI* and *Sall* ends onto C99. PCR was also used to amplify C99 from an SFV vector containing full-length human APP751 (a kind gift from Dr. R. Doms, University of Pennsylvania). Since APP751 had an internal *BamHI* site, we first performed site directed mutagenesis (Invitrogen) using the primers APPBamF and APPBamR (see Table 1 for all primer sequences) to remove this internal site. Then, using the primers C99Xba2 and *Sall* (see Table 1) PCR was used to amplify and isolate C99 from the SFV751 vector. The PCR products were purified using PCR column chromatography (Qiagen) and agarose gel purification. Ligation of C99 into the JJB20 backbone was followed by gel purification. Confirmation that C99 was inserted into the vector was obtained upon sequencing the new construct using the MF1 primer (see Table 1). This primer anneals to a region in the MF1 α pre pro construct, ~200 bp upstream from the *XbaI* C99 insertion site. The sequence of the inserted C99 was confirmed using DNA sequence analysis.

C99 degradation in yeast

A 3-ml culture of C99-expressing wild type (*PRE1–PRE2*) or proteasome mutant (*pre1-1pre2-1*) yeast cells were grown overnight in SC–ura +2% raffinose at 26 °C. The cells were then inoculated to an initial concentration of 0.2 OD (optical density units at 600 nm) in a total volume of 10 ml of SC–ura +2% galactose and allowed to grow to saturation overnight. The next day the cells were inoculated to an initial concentration of 0.3 OD in 25 ml of SC–ura +2% galactose and allowed to grow for 2 h at 26 °C. A total of 10 ODs of each strain were harvested and resuspended to a final concentration of 4 OD/ml. The cultures were shifted to 37 °C for 10 min, the protein synthesis inhibitor cycloheximide (50 μ g/ml) was added, and 1.4 ODs of cells were harvested and added to sodium azide (100 mM final concentration) at the indicated time points. The cells were recovered in a microcentrifuge and the pellet was resuspended in 200 μ l of ice-cold extract buffer (10 mM Tris–HCl, pH 8, 25 mM ammonium acetate, 500 mM iodoacetamide, 20 mM sodium azide, 20 mM EDTA, 10% trichloroacetic acid, 0.45 mM cycloheximide) to which protease inhibitors had been freshly added (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 3 μ g/ml leupeptin). Zirconium oxide beads were added to just below the meniscus and the mixture was agitated on a Vortex mixer on the highest setting for 45 s. The supernatant was removed, mixed with 1x lithium dodecyl sulfate–PAGE sample buffer (Invitrogen) that contained 10 mM dithiothreitol, and heated at 70 °C for 10 min. Proteins were resolved using 4–12% Bis–Tris gels (Invitrogen) and transferred to

nitrocellulose for immunoblot analysis. The desired proteins were detected using D4253 antiserum, which was generated using the same C-terminal peptide sequence as that used to generate the anti-A β C-terminal 5685 and 2493 antibodies [45]. The bound antibody was detected using anti-rabbit-horseradish-peroxidase-conjugated secondary antibody and the signal was developed using the picomole sensitive Super Signal kit (Pierce).

Zip Tip immunocapture and mass spectrometry

A control experiment was first performed to verify that A β peptides could be separated prior to mass spectrometry analysis using antibody 6E10 (purchased from Sigma), which targets amino acids 1–17 of the A β sequence [46]. Any fragments generated from C99 containing all or most of the A β 1–17 epitope will be recognized by the 6E10 antibody. Peptide standards, bovine serum albumin (BSA), solvents, and matrix compound were purchased from Sigma–Aldrich. A binding-control mixture of two peptides that are bound with 6E10 (A β 1–40 and A β 1–42) and two non-binding peptides (angiotensin I and adrenocorticotrophic hormone clip 18–39) was made with each peptide at 1.0 μ M. A total of 0.3 μ g of 6E10 antibody was taken up into a C18 Zip Tip (Millipore), which was then blocked by repeated elution with 10 mg/ml BSA. This elution step binds BSA to the available hydrophobic surfaces on the tip. The peptide mix was taken-up into the tip, which was then washed with ammonium citrate buffer (20 mM, pH7.4) and water to remove nonspecifically bound peptides. To elute bound peptides, 3 μ l of 1% trifluoroacetic acid [47] was added. This TFA elution was then taken up into another C18 Zip Tip (with no bound antibody), desalted with 0.1% TFA washes, and eluted onto a sample plate with 2 μ l of matrix solution (50% acetonitrile (MeCN), 0.1% TFA, 5 g/L α -cyano-4-hydroxycinnamic acid (CHCA)).

All spectra were taken on an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer with no guide wire. Both reflector and linear modes were used. CHCA was used as the matrix, and a positive accelerating voltage of 20 kV and delayed extraction were employed. Four hundred laser shots were averaged to obtain each spectrum. The instrument settings for reflector mode were grid voltage = 66.5% and delay = 400 ns. The linear mode settings were grid voltage = 95% and delay = 575 ns. Mass spectrometric analysis detected both of the A β peptides (with isotopic resolution under reflector mode) and neither of the control peptides (data not shown).

Having established that A β peptides could be isolated using Zip Tip MALDI-TOF technology, 1 L of 0.13 OD/ml C99-expressing *PRE1PRE2* cells and 1 L of 0.2 OD/ml C99 expressing *pre1-lpre2-1* cells were grown, harvested, and resuspended in 2 ml of extract buffer, and a yeast cell lysate was prepared as described above. A total of 500 μ l of the lysate was then filtered through a Microcon spin filter (10-kDa molecular weight cutoff; Millipore). The 6E10 antibody was again bound to a C18 Zip Tip and the filtered cell lysate was taken up in the Zip Tip repeatedly over 5 min. The antibody-conjugated Zip Tip was washed and eluted as above, and this eluate was desalted on another Zip Tip (lacking antibody). Bound peptides were eluted and mixed with matrix as described above. The linear mode on the mass spectrometer was used to analyze these samples to increase the sensitivity for low-abundance peptides, while reflector mode was used for better mass accuracy.

The detected peaks are listed (Tables S1, S2, and S3) as monoisotopic (reflector) or average (linear) m/z values. The exceptions are reflector peaks 5037 (*pre1-lpre2-1*) and 4827 (*PRE1PRE2*), which were Gaussian smoothed and given as an average mass, since no monoisotopic peak could be resolved. The major peaks for peptides under these conditions will be from singly charged ions of the form MH^+ . In these experiments no evidence of higher charge states was detected. This was confirmed by examining the mass difference between isotope peaks in reflector mode. The mass difference between the detected peaks and the theoretical masses of C99 fragments are given, along with the amino acid range and the

sequence itself. The amino acids immediately before or after the sequence are given in parentheses, and these were used to assign the peptide type. Since it is uncertain whether the N-terminal Met is present when C99 is expressed (see Fig. 1), this residue is assigned to zero so that the rest of the sequence follows the standard A β numbering.

Various fragments of the A β peptide were detected in both samples (Tables S1, S2, and S3) including peptides matching the masses of A β 1–44 and A β 1–45. The mass uncertainties are due to the decreased accuracy of the linear mode. Several A β fragments have the same composition (even though the sequence is a different order), and thus the masses are identical. Tables S1–S3 list all possible sequences that match a given mass.

Results and discussion

The stability of C99 is controlled by the proteasome in the yeast *S. cerevisiae*

To ensure that C99 would traverse the secretory pathway we chose to clone the sequence encoding C99 into a construct that had been previously used to trigger the entry of APP into the *S. cerevisiae* secretory pathway [34]. The gene was placed under the control of a galactose-regulated promoter. In addition, the N terminus of the protein contains the pre pro sequence from the yeast α factor mating pheromone, a sequence that is known to lead to the efficient targeting of nascent polypeptides into the endoplasmic reticulum and through the secretory pathway (Fig. 1) [34]. Notably, the proteasome is known to affect the quality control of proteins that enter the secretory pathway [48] and, as described above, has been suggested to affect APP processing.

To determine whether proteasome activity directly impacted the stability of C99 in yeast, the degradation of this protein was examined by immunoblot analysis from extracts from wild-type (*PRE1PRE2*) and proteasome mutant (*pre1-1pre2-1*) strains after the addition of cycloheximide. Although the genes encoding proteasomal subunits cannot be deleted, the *pre1-1pre2-1* strain utilized in this study leads to the absence of ~95% of the proteasome's activities [43]. The results of this cycloheximide-chase experiment indicated that the absence of proteasomal activity had profound effects on two C99-generated products (Fig. 2). These products were detected using the D4253 antibody, which recognizes the C terminus of the A β peptide [45]. These data demonstrate that the proteasome processes C99-derived, C-terminal fragments of ~8 and ~10 kDa in mass.

Complete fragment profiles generated from C99 are radically different in the presence and absence of proteasome activity

While the role of the proteasome has been indirectly investigated in mammalian cells using pharmacological agents [49], the use of these agents can induce nonspecific stress responses. We have been able to surmount this potential problem. Furthermore, we wanted to identify the C99-derived fragments generated in the absence of a functioning proteasome but in the presence of other cellular quality control machineries. Since a failing proteasome may be responsible for the aggregation of fragments of APP in the AD brain [22] we wanted to identify the full spectrum of the generated fragments. We believed that this may also provide clues about the types of C99-generated fragments in mammalian cells, in the absence of the proteasome. We believed further that this information would also allow us to decipher the compensatory processes that occur in the absence of this protease complex and provide some insights as to what possibly happens in the AD brain. Hypotheses generated from this undertaking could then be tested in mammalian cells.

Because of the strong degree of stabilization of (at least) two C99-derived fragments in the absence of proteasomal activity, and because the processing of C99 can generate aggregation-

prone fragments that lead to AD (see above), the identities of C99 fragments produced in wild-type (*PRE1PRE2*) and proteasome-deficient (*pre1-1pre2-1*) yeast were examined. To this end, C99-expressing wild-type and proteasome mutant yeast lysates were prepared and processed as described under Materials and methods. In brief, Zip Tips were used to immunocapture C99-generated fragments (with an antibody generated against amino acids 1–17 of the A β peptide) and MALDI-TOF mass spectrometry was used to determine the identities of the species that had bound to the matrix. Over one dozen peptide fragments from wild-type yeast cells expressing C99 (Figs. 3A and S1) and three dozen fragments from proteasomal mutant yeast cells expressing C99 (Figs. 3B, S2, and S3) were identified using reflector and linear modes.

Since secretases, neprilysin, insulysin, and other proteases, have been identified as mediators of A β degradation *in vivo* [50,51] (Fig. 3C), we next wanted to classify the fragments into categories potentially produced by one enzyme versus another. To make our findings more comprehensible we divided the identified fragments into subtypes (Table 2). Type A comprises large fragments that appeared to be derived from a secretase-like activity on C99. Types B and C comprises smaller fragments generated from Type A fragments by a NEPLP activity in addition to the secretase-like activity. Type D comprises smaller fragments than Type A. The Type D fragments were not produced by NEPLP cleavage but may have derived from secretase-like activity. However, NEPLP activity cannot be ruled out in this case since the fragments might have been derived from Type B and C fragments. Since some of the Type D peaks have masses similar to those of as the Type B or C peaks, they have been labeled *D. In the Type *D peaks, NEPLP activity cannot be ruled out. The specific peaks detected by mass spectrometry are listed in Table 3A and B.

Since yeast lack homologues for NEP we proposed that insulysin homologues may substitute for the lack of proteolytic processing by the proteasome. Insulysin, like NEP, preferentially cleaves the N-terminal side of a bulky hydrophobic residue in small peptide substrates (<40–60 residues) [19,28,29] and has activity against A β *in vivo* [19]. The presence of homologues of insulysin enzyme in yeast (the pitrilysin endopeptidases Axl1p and Ste23) [41] along with the identities of several fragments {13–31, 13–33, 13–42, 15–51, 4–29, 2–15, 1–20} (Tables 3B, S2, and S3) makes this a likely candidate for the processing of the C99 in this system. For example, this enzyme can cleave A β *in vitro* to form a 1–20 fragment that is present in the *pre1-1pre2-1* cells expressing C99 in this study (Tables 3B, S2, and S3). Because none of these fragments are detected in *PRE1PRE2* cells expressing C99 we suggest that there is a compensatory activity for an NEPLP- or insulysin- type activity in mutant *pre1-1pre2-1* cells that lack proteasomal activity.

Based on the fragments generated in the wild-type and proteasome-deficient yeast cells expressing C99, the activities of many cellular enzymatic activities—along with the proteasome and the vacuole (the lysosome equivalent in yeast)—can be postulated to play roles in the processing of C99. Specifically, in wild-type cells we observed intact A β fragments 44 and 45 amino acids long. We also see fragments in these cells that end between residues 42 and 55. The presence of these intact A β fragments (A β 1–44 and A β 1–45) only in the wild-type sample suggests the role of the proteasome in the generation of longer and more aggregation-prone peptides. Another possibility is that yeast cells express a “sloppy” ϵ [52–54] or ζ secretase-like protease [55] (Fig. 3C). It should also be noted that although protease inhibitors were added to all buffers utilized and all lysates were kept at 4 °C to minimize protease activities during handling, *ex vivo* lysis cannot be completely excluded.

In the *pre1-1pre2-1* cells, there is evidence of intact A β peptide and a change in the population of fragments formed. Due to the presence of many small fragments in the mutant cells expressing C99, we propose that defects in proteasome function allow C99 and C99-derived peptides to become substrates for other cellular enzymes. The increased number of fragments

generated in the absence of the proteasome that possess either one or both of two β -stranded regions (10–22 and 30–35 in the A β peptide) is known to increase the propensity of proteins/peptides to aggregate. This is even more interesting when we consider that some of the fragments generated from C99 resemble those detected in the brain and cerebrospinal fluid (CSF) of AD patients [3,5,56]. In brains from Alzheimer's patients, for example, 6 times more water-soluble N-42 type fragments (i.e., fragments starting at the β -secretase cleavage site or further upstream and referred to as “ragged”) have been identified, and 50 times more N-42 is present in AD CSF compared to controls [56]. In addition, similar to those identified in this study, the purification of amyloid fragments from senile plaques of Alzheimer's patients unveils the presence of many APP fragments with “ragged NH₂ termini” [57] or C-terminally truncated forms (i.e., cleavage that result in fragments before the normal position 40/42) of APP peptides in the CSF of Alzheimer's patients [58]. Thus, we believe that our findings are physiologically relevant to the situation occurring in the human AD population.

Our work also suggests that compensatory processing of C99 by other cellular enzymes readily takes over when proteasome activity is decreased, as occurs when humans age [22]. This compensatory response leads to the generation of products that are more hydrophobic and aggregation prone and hence could cause an increased rate of progression of neurodegenerative disease. As noted above, this result may be physiologically relevant: Monomeric, dimeric, and small diffusible versions of A β -derived diffusible ligands have been found to be toxic when examined in several mammalian experimental systems (e.g., when effects on long-term potentiation are examined) [59,60].

That the wild-type cells expressing C99 exhibit an overall reduced number of fragments (see Tables 3A and S1 versus 3B, S2 and S3) and that the fragments present in wild type cells have C termini that end at amino acid 17 suggest the existence of enhanced, non toxic α -secretase cleavage (4–17, 1–17). This result hints that a functioning proteasome may be protective against the formation of aggregation prone hydrophobic fragments. The exceptions to this statement are the two longer fragments found in wild-type cells expressing C99 (Tables 3 and S1). The presence of these two fragments suggests the potential for the proteasome to be involved in amyloidogenic-type processing but with most of the proteasomal processing still producing nonamyloidogenic fragments. Whether these two longer fragments are generated directly by the proteasome or by other cellular enzymes remains, however, to be elucidated and represents the topic of future efforts. In any event, the correlation between what has been found in Alzheimer's patients and in this study points to the increased “toxicity” that can be induced by compromised proteasomal activity and to the compensatory processing in neuronal cells that can lead to an accelerated development of neurodegenerative diseases, such as Alzheimer's disease. In addition to this important finding, this study provides further justification for the use of yeast proteomic methods to model the molecular defects in specific disease states.

Appendix A. Supplementary data

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. C99 expression vector and encoded sequence

Vector used for the expression of C99 in the yeast *S. cerevisiae*. “Gal” denotes the galactose-inducible promoter, “pre” and “pro” refer to the region cloned from the pre pro α factor mating pheromone in yeast. “Amp” and “URA3” refer to genes for growth selection in *E. coli* and yeast, respectively. “2 μ ” is for multicopy maintenance in yeast. The DNA sequence and the resulting amino acid sequence (in single letter code) of human C99 are also included. pBM258 and PMF refer to the sequences obtained by PCR that were used for plasmid construction (see Materials and methods).

Fig. 2. The proteasome degrades C99-generated polypeptides in yeast

Cycloheximide-chase experiments were performed on C99-expressing wild-type (*PRE1PRE2*) and proteasome mutant (*pre1-1pre2-1*) yeast strains as described in the Materials and methods. Protein extract from equal numbers of cells were loaded at each time point and for each strain. The ~10- and ~8-kDa fragments are stabilized upon the removal of proteasomal activity.

Fig. 3. Fragments generated from C99 in both the presence and the absence of proteasome activity as assessed after immunocapture and MALDI-TOF mass spectroscopy

MALDI-TOF mass spectra of Zip-Tip-captured C99-derived peptides from (A) wild-type and (B) proteasome mutant cells taken in reflector mode with CHCA as the matrix after baseline correction, noise removal, and Gaussian smoothing. No MS/MS sequencing (post-source decay) could be done on the peaks since the absolute intensities were too low for peaks in the given mass range. (C) Cellular enzymes proposed to be involved in C99 processing, including the secretases (α , β , γ , ζ , ϵ) and/or insulysin/NEPL and/or the vacuolar proteases.

Table 1

Primers used in this study

Primer name	Primer sequence
<i>APPBamF</i>	5'gagcatgtgcgcatggtggacccaagaagccgctcag3'
<i>APPBamR</i>	5'ctgagcggcttcttgggtccaccatgcgcacatgctc3'
<i>C99Xba2</i>	5'ctatctctagataaaagaatggatgcagaattccgacatgac3'
<i>Sall</i>	5'aaggctgttgcgacctagtctgcacatgctcaa3'
<i>MF1</i>	5'gttttattcgagcatcctccgc3'

Table 2

Fragment classification for NEPLP- generated fragments from C99

Type	Characteristics
A	Contains the N-terminal 38 to 55 residues of C99, probably result of secretase only
B	Hydrophobic head, possibly result of NEPLP cleavage on its N-terminal side
C	Fewer than 38 residues, and residue immediately after C terminus is hydrophobic, possibly result of NEPLP cleavage there
BC	Has characteristics of both Types B and C
D	None of the above
*D	As Type D, but mass similar to that of a fragment that is Type B or C, therefore NEPLP activity is still possible

Table 3
Sequences of fragments that match peaks detected in the MALDI-TOF spectra taken in reflector mode of (A) *PRE1PRE2* and (B) *pre1-pre2-1* yeast strains expressing C99

<i>m/z</i> expr.	<i>m/z</i> theo.	Delta	Start	End	Type	Sequence
A						
1590.8927	1590.7911	0.1	14	27	D	(H) HQKLVFFAEDVGSN (K)
1590.8927	1590.8063	0.1	8	20	C	(D) SGYEVHHQKLVFF (A)
1752.1248	1752.8565	-0.7	4	17	BC	(E) FRHDSGYEVHHQKL (V)
1818.1444	1818.8810	-0.7	9	23	BC	(S) GYEVHHQKLVFFAED (V)
2068.5380	2067.9631	0.6	1	17	C	(M) DAEFRHDSGYEVHHQKL (V)
2379.9910	2380.2772	-0.3	13	34	C	(V) HHQKL VFFAFDVGSNKGAIIGL (M)
2401.9996	2401.1207	0.9	6	26	D	(R) HD SGYEVHHQKLVFFAEDVGS (N)
2715.4686	2715.3889	0.1	9	33	BC	(S) GYEVHHQKLVFFAEDVGSNKGAIIG (L)
2802.6119	2802.4210	0.2	8	33	C	(D) SGYEVHHQKLVFFAEDVGSNKGAIIG (L)
4827.8697	4826.6539	1.2	4	48	BC	(E) FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL M VGGVVIATVIVIT (L)
4827.8697	4828.5389	-0.7	1	45	A	(M) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL M VGGVVIATVI (V)
B						
1935.9750	1936.0538	-0.1	15	32	C	(H) QKLVFFAEDVGSNKGAIIG (G)
2461.2165	2461.1684	0.0	1	20	BC	(M) DAEFRHDSGYEVHHQKLVFF (A)
2461.2165	2461.1684	0.0	4	23	BC	(E) FRHDSGYEVHHQKLVFFAED (V)
2902.4483	2902.4920	0.0	10	35	BC	(G) YEVHHQKLVFFAEDVGSNKGAIIGL (V)
2907.4903	2907.3155	0.2	0	23	C	(-) MDAEFRHDSGYEVHHQKLVFFAED (V)
3426.6260	3426.8242	-0.2	10	41	BC	(G) YEVHHQKLVFFAEDVGSNKGAIIGL M VGGVVI (A)
3807.8657	3808.1809	-0.3	14	50	C	(H) HQKLVFFAEDVGSNKGAIIGL M VGGVVIATVIVITLV (M)
4611.8448	4611.5081	0.3	8	51	C	(D) SGYEVHHQKLVFFAEDVGSNKGAIIGL M VGGVVIATVIVITLV (L)
5037.8554	5037.6295	0.2	1	47	A/D	(M) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL M VGGVVIATVIVIT (T)
5037.8554	5037.8763	0.0	16	62	D	(Q) KLVFFAEDVGSNKGAIIGL M VGGVVIATVIVITLV M L K K K Q Y T S I H H (G)

Fragments were verified in reflector mode by accepting only peaks that had two distinct isotope peaks beyond the monoisotopic peak with a signal to noise ratio greater than 40:1. Experimentally determined masses are given, along with the theoretical mass of the sequence and the difference ("delta") in Daltons. The peptide type, numbering, and sequence are as described in the text.