Processing of N3, a mammalian proteasome beta-type subunit

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Proteasome subunits are encoded by members of the same gene family and can be divided into two groups based on their similarity to the alpha and beta subunits of the simpler proteasome isolated from *Thermoplasma acidophilum*. RN3 is the beta-type subunit, N3, of rat proteasomes which has been implicated in the peptidylglutamyl-peptide hydrolase activity of the proteinase complex. We have expressed recombinant RN3 protein in *Escherichia coli* in order to raise subunit-specific polyclonal antibodies. Identification of the position of RN3 on two-dimensional PAGE gels of purified rat liver proteasomes showed a single protein spot of molecular mass 24 kDa and of pI value of about 5. This protein has a free N-terminus, having undergone post-translational processing. After immunoprecipitation from [^{35}S]methionine-labelled human embryo lung L-132 cells using anti-RN3 antibodies, two radiolabelled spots were observed on two-dimensional PAGE gels, one corresponding to the mature N3, the other of molecular mass 28.5 kDa and pI value around 5, which was probably the unprocessed form of N3. However, the latter protein had a higher molecular mass (31 kDa) than was predicted from the sequence of previously cloned cDNA. Therefore rapid amplification of cDNA ends ('RACE') was carried out to determine the full sequence. The lack of detectable RN3 precursor in purified rat liver proteasomes suggests that the processing probably accompanies assembly of the complex. The half-life of the processing was determined to be 31 min in growing L-132 cells. The unprocessed form of N3 was not observed after immunoprecipitation of ^{35}S-labelled complexes with anti-proteasome antibodies. There was no evidence to suggest that unprocessed N3 is found in precursor complexes which have been implicated in the assembly of some other unprocessed beta-type subunits. Interestingly also, the site of cleavage of N3 (ITR↓TQN) differs significantly from those of other processed animal beta-type proteasome subunits [(H/T)G↓TT(T/L)], many of which resemble more closely the cleavage site of the *Thermoplasma acidophilum* beta subunit.

INTRODUCTION

Proteasomes (multicatalytic proteinase complexes) are high-molecular-mass (700 kDa) multimeric proteinase complexes which constitute the major non-lysosomal degradative machinery of eukaryotic cells [1–3]. They are found in the nucleus and cytoplasm [4] and can be purified either by themselves or as part of the 26 S proteinase complex, which plays an important role in both ubiquitin-dependent and ubiquitin-independent protein degradation. The cylindrical structure of proteasomes is made up of four stacked rings of seven subunits each. In a simple form of the proteasome which has been isolated from *Thermoplasma acidophilum*, there are only two different types of subunits: alpha subunits, which form the outer rings, and beta subunits, which form the inner rings of the cylindrical structure [5]. Eukaryotic proteasomes are composed of at least 14 different types of subunit (22–34 kDa) which are encoded by members of the same gene family. These subunits can be divided into two groups based on their similarity to the archaebacterial alpha and beta subunits. The structure of eukaryotic proteasomes shows two-fold symmetry and is similar to that of the archaebacterial enzyme, except that the rings are each composed of non-identical subunits [6]. Yeast proteasomes appear to have seven different alpha and seven different beta subunits, all of which have now been cloned [7]. Animal cell proteasomes contain homologues of the yeast proteasome subunits, but there are additional beta-type subunits. Two non-essential proteasome genes encoding beta-type subunits, *LMP2* and *LMP7*, are located in the major-histocompatability-complex (MHC) class II region and have been linked to a role for proteasomes in antigen presentation by the MHC class I pathway [8,9].

One characteristic of the beta-type subunits, which have a catalytic function [9], is that most of them are processed at the N-terminus. Seven free N-terminal sequences were determined for rat liver proteasomes [10] and five for human erythrocyte proteasomes [11]. We have previously cloned one of the processed rat proteasome beta subunits, RN3 [12]. The yeast homologue of RN3 has been implicated in peptidylglutamyl-peptide hydrolase activity of the complex, because mutants lacking the C-terminal 15 amino acids are defective in this activity [13]. The N3 subunit is particularly interesting because it is not closely related to the other beta-type subunits, and the site of cleavage of the proprotein is different from that of the other beta subunits, including the beta subunit of the *Thermoplasma* proteasome.

In the present study we have expressed recombinant N3 protein for the production of subunit-specific antibodies to investigate the distribution and processing of this subunit. Our results support the view that N3 is present in all proteasomes and show that the characteristics of its processing are different from those reported for the non-essential MHC-encoded subunits [14–16]. Our results question the hypothesis that the proproteins are present in precursor complexes [16]. The complete sequence of the propeptide was obtained by 5′ **r**apid **a**mplification of **c**DNA **e**nds (RACE). Comparison of the sequence with that of other beta subunits suggests a different mechanism of cleavage, possibly involving a different type of catalytic activity.

MATERIALS AND METHODS

Materials

Nonidet P40, 2-mercaptoethanol, sodium deoxycholate, Protein A–agarose, 5-bromo-4-chloroindol-3-yl phosphate, Nitroblue Tetrazolium, Triton X-100, Tween 20, dithiothreitol and Ponceau

Abbreviations used: IEF, isoelectric focusing; RACE, **r**apid **a**mplification of **c**DNA **e**nds; MHC, major histocompatability complex; (M-)DMEM, (methionine-free) Dulbecco's modified Eagle's medium.

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S were from Sigma Chemical Co. Restriction enzymes and other reagents for molecular biology were purchased from Pharmacia, except for reverse transcriptase, which was from Promega. Dulbecco's modified Eagle's medium (DMEM), methionine-free Dulbecco's modified Eagle's medium (M-DMEM) and newborn-calf serum were purchased from ICN Flow. Trypsin/EDTA in Puck's saline, and penicillin/streptomycin (5000 i.u./5000 μg per ml) were supplied by GIBCO BRL. Acrylamide/bisacrylamide (75:2, w/v) (Protogel) was from National Diagnostics, and Coomassie Brilliant Blue R-250, low molecular weight protein standards were from Bio-Rad Laboratories. Affinity-purified pig anti-rabbit IgG–alkaline phosphatase conjugate was obtained from Dakopatts, dried milk powder (Marvel®) from Cadbury, cellulose nitrate paper (0.1 μm pore size) from Schleicher and Schuell, SDS (ultra pure) and urea (ultra pure) from United States Biochemical, ampholytes, pH 3.5–10 (Electran grade) from BDH, and [^{35}S]methionine (37 TBq mmol) was from Amersham International. All other reagents were of analytical grade. Rat liver proteasomes were purified as described in [17].

Expression of RN3

RN3 was expressed in *Escherichia coli* using the QIAexpressionist system (QIAGEN). The vector used was pQE12, which gives a recombinant protein with a 6 × His tag at the C-terminus. RN3 cDNA [12] was amplified from the SK+ vector by PCR using primers based on the 5′ and 3′ ends of the cDNA, but incorporating *Bam*HI and *Bgl*II into sense and antisense primers respectively {sense primer: $^{5'}$GGGGATCCATGTCTGCGCCT$^{3'}$ (positions 10–21 of cDNA sequence [12]); antisense primer: $^{5'}$GGGAGATCTTTCAAAGCCAC$^{3'}$ (positions 685–695 of cDNA sequence [12]}. After cloning of the cDNA into the vector and identification of recombinants, the plasmid was sequenced [18] at the 5′ and 3′ ends of the cDNA to check that it was in-frame with the initiation codon and the 6 × His tag respectively. Recombinant RN3 protein was expressed in exponentially growing *E. coli* XL1-Blue cells following induction with 3 mM isopropyl β-D-thiogalactoside.

The expressed protein was purified by Ni-chelating chromatography [19] followed by reverse-phase HPLC using a gradient of 0–70 % acetonitrile in 0.1 % trifluoroacetic acid.

Production of anti-RN3 antibodies

Polyclonal antibodies were raised against the purified RN3 protein in a New Zealand White rabbit (University of Leicester Biomedical Unit) by injecting antigen (200 μg for the primary injection, 100 μg for subsequent injections, in PBS) intravenously every 5 days over a 2-month period. Polyclonal anti-(rat liver proteasome) antibodies were those described previously [20]. Purified IgG preparations (pre-immune, anti-proteasome, anti-RN3) were obtained using Protein A–agarose, and affinity purification of anti-RN3 IgG was carried out using rRN3 protein as described for other antibodies [21].

PAGE and immunoblot analysis

SDS/PAGE was carried out by the method of Laemmli [22] using a 15 % separating gel. Two-dimensional electrophoresis was carried out by isoelectric focusing (IEF) and SDS/PAGE according to a modified method of O'Farrell [23].

Immunoprecipitated material was eluted from the Protein A–agarose-bound antibody for SDS/PAGE by adding SDS sample buffer to the gel and boiling for 5 min, or for IEF by adding 40 μl of 9 M urea to the pellets and incubating for 5 min on ice before adding 30 μl of purified rat liver proteasomes (1 mg/ml) and incubating for a further 10 min. The gel was sedimented and the supernatants removed to clean tubes. IEF sample buffer [30 μl of 2 % (w/v) ampholytes, pH 3.5–10, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 % (v/v) Triton X-100, 5 mM ascorbic acid and 10 mM dithiothreitol] was added to each sample before loading them on the IEF rods.

Proteasome subunits were electrophoretically blotted to nitrocellulose sheets using a Bio-Rad Trans-Blot apparatus with 25 mM Tris/192 mM glycine/20 % (v/v) methanol as the transfer buffer. Transferred proteins were detected with 0.2 % Ponceau S in 3 % (w/v) trichloroacetic acid and immunoblotting was carried out as described previously [21] using alkaline phosphatase-conjugated secondary antibodies with 5-bromo-4-chloroindol-3-yl phosphate and Nitroblue Tetrazolium.

Cell culture

Rat-1wt cells (Rat-1 fibroblasts transformed with Rous sarcoma virus) and human embryonic lung (L-132) cells were routinely grown in DMEM supplemented with 10 % (v/v) newborn-calf serum and penicillin/streptomycin (50 i.u./ml and 50 μg/ml respectively) in a humidified atmosphere of CO_2/air (1:19).

[^{35}S]Methionine labelling of cellular proteins and immunoprecipitation of N3

Cells were grown in 25 cm^2 plastic culture flasks (Nunc, Roskilde, Denmark). For labelling with [^{35}S]methionine, the medium was removed from subconfluent cells and the cells rinsed briefly with PBS. Cells were then incubated for 60 min in M-DMEM supplemented with 10 % (v/v) newborn-calf serum and antibiotics. The medium was then removed from the flasks and 2 ml of fresh M-DMEM and 100 μCi of [^{35}S]methionine (7.5 μl) placed in each flask.

After labelling, the medium was carefully removed from the culture flasks, which were then rinsed three times with PBS. A 1 ml portion of ice-cold RIPA buffer [50 mM Tris (pH 8.0)/150 mM NaCl/1 % (v/v) Nonidet P40/0.2 % SDS/0.5 % sodium deoxycholate] was added directly to the culture flasks. The flasks were placed on ice for 30 min with occasional gentle mixing to ensure complete cell lysis. The lysates were removed to Eppendorf tubes, sonicated (20 μ power for 10 s), heated at 94 °C for 4 min and then centrifuged for 5 min to sediment any insoluble material.

Preimmune IgGs were bound to Protein A–agarose at 400 μg of protein/200 μl of swollen gel in NET buffer [50 mM Tris/HCl (pH 7.0)/150 mM NaCl/5 mM EDTA/0.1 % BSA/0.1 % SDS/0.1 % sodium deoxycholate/0.5 % (v/v) Nonidet P40]. Immunoprecipitation with polyclonal anti-proteasome IgG was carried out as described previously [20].

After SDS/PAGE or two-dimensional PAGE and electroblotting of immunoprecipitated material, the nitrocellulose was exposed to Kodak X-OMAT AR film at −70 °C to reveal [^{35}S]methionine-labelled proteins.

Northern blotting

Total RNA was prepared from fresh rat tissue using the single-step method of Chomczynski and Saachi [24]. The yield and quality of the RNA were determined by spectrophotometry and agarose-gel electrophoresis. RNA (30 μg) was separated by agarose-gel electrophoresis and blotted on to Hybond-N (Amersham) using standard procedures [25]. A 400 bp *Eco*R1/*Hin*dIII fragment of RN3 cDNA was labelled using [α-^{32}P]dATP and then used as a probe for hybridization using standard protocols [25]. Kodak X-AR film was exposed to the membrane at −70 °C.

RACE

5′ RACE was performed following the procedure of Frohman [26]. Total RNA (2 μg) extracted from rat fibroblasts (Rat-1wt) was used for first-strand cDNA synthesis using the antisense primer: $^{5'}$GGGAAGCTTTCTCCAC$^{3'}$ (positions 385–401 of cDNA sequence [12]). A poly(dA) tail was added to the cDNA using dATP with terminal transferase at 37 °C for 2 h. The resulting cDNA was used as a template for PCR with a sense primer, incorporating a *Hin*dIII restriction site: $^{5'}$CCCGGGAAGCT$_{(20)}{}^{3'}$ and an antisense primer, incorporating an *Eco*R1 restriction site: $^{5'}$GGGAATTCCGAGAACTTGCTT$^{3'}$ (positions 229-242 of cDNA sequence [12]). The RACE product was purified, cloned and sequenced from five separate colonies following standard protocols [25].

RESULTS

Expression of RN3 from the pQE12 vector on induction with isopropyl β-D-thiogalactoside resulted in the production of two proteins (molecular masses 24 and 25 kDa) in *E. coli* extracts. DNA sequencing confirmed that the RN3 cDNA was correctly inserted in the vector at both the 5′ (DNA sequence encodes a protein sequence MRGSMSA-; MRGS from the vector sequence, MSA from the RN3 sequence), and 3′ (DNA sequence encodes a protein sequence -GFEHHHHHH) ends. N-terminal sequence analysis of the protein bands electroblotted on to poly(vinylidine difluoride) membrane identified the major band (24 kDa) as recombinant RN3 (determined sequence MRGSM, as would be predicted). RN3 was found mostly in the insoluble fraction and was therefore purified using the denaturing purification protocol.

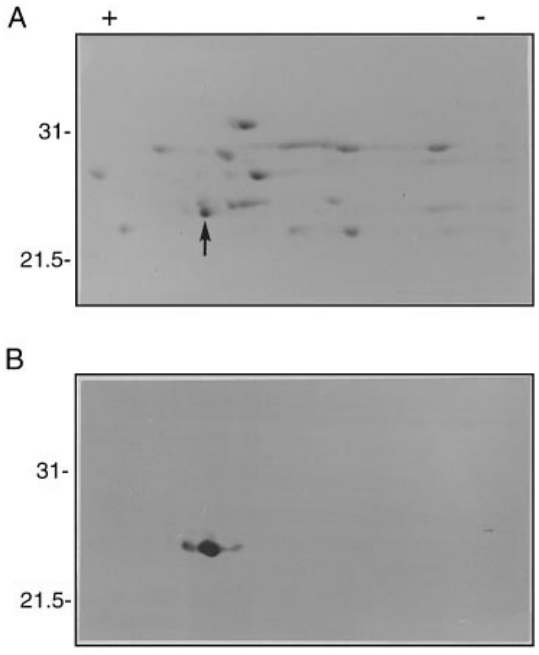

Figure 1 Two-dimensional PAGE gels of purified rat liver proteasomes immunoblotted with anti-RN3 antibodies

(**A**) Purified rat liver proteasomes (30 μg) were run on an IEF rod gel and then electrophoresed on an SDS/PAGE gel, electroblotted on to nitrocellulose membrane and then stained with Ponceau S. The arrow (↑) indicates the position of the subunit identified in (**B**). Molecular mass markers, in kDa, are indicated in the left. (**B**) The nitrocellulose membrane from (**A**) was probed with anti-RN3 polyclonal antibodies and developed as detailed in the Materials and methods section.

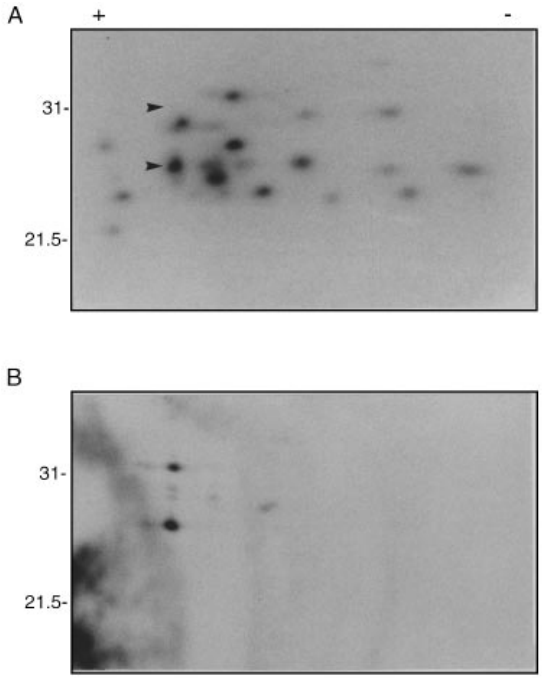

Figure 2 Immunoprecipitation of proprotein with anti-RN3, but not anti-proteasome, polyclonal antibodies

Human embryo lung (L-132) cells were labelled with [^{35}S]methionine for 1 h, and proteasomes were immunoprecipitated as described in the Materials and methods section. Samples were run on two-dimensional PAGE gels. (**A**) Autoradiogram of material immunoprecipitated with anti-proteasome antibodies. The arrowheads indicate the positions of spots in (**B**). The positions of molecular-mass markers, given in kDa, are indicated on the left. (**B**) Autoradiograph of material immunoprecipitated with anti-RN3 polyclonal antibodies.

Recombinant proteins produced with their 6 × His tag using the QIAexpressionist system can normally be purified in a single step using a column of chelating Sepharose charged with Ni^{2+} [19]. However, rRN3 was not bound to the column and so the flow through from the Ni–Sepharose column was further purified by reverse-phase HPLC to give an apparently homogeneous rRN3 preparation, as judged by SDS/PAGE. Since DNA sequencing of the vector confirmed correct insertion of the RN3 cDNA, the full construct, including the six histidine residues, should have been expressed. However, it is possible that the 6 × His tag was lost by cleavage of the expressed protein, which would also explain the observed molecular mass of 24 kDa compared with the expected value of 27 kDa. The purified protein was used to raise subunit-specific polyclonal antibodies.

Northern-blot analysis suggested that the N3 gene, expressed as a 1.2 kb transcript, was expressed to approximately equal levels in a variety of rat tissues (results not shown). This information, together with Western-blot analysis of N3 levels, which paralleled those of proteasomes detected using anti-proteasome antibodies [21], suggest that N3 is a constitutive subunit of the eukaryotic proteasome complex. RN3 was found to be present in both purified rat liver proteasome and 26 S proteinase preparations (results not shown).

Immunoblot analysis of purified rat liver proteasomes with anti-RN3 antibodies allowed identification of the position of the RN3 protein on two-dimensional PAGE gels and showed that its size was that predicted for the N-terminally processed form of

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                                5'TTTTTTTTTTTTTTTTTTTTCGGCAGTGGTGACTAAG   -1
 M  E  A  F  W  E  S  R  T  G  H  W  A  G  G  P  A  P  G  Q              20
ATGGAAGCGTTTTGGGAGTCACGGACTGGCCACTGGGCCGGGGGTCCGGCTCCGGGGCAG  60
 F  Y  R  V  P  S  T  P  S  C  L  M  D  P  M  S  A  P  A  R              40
TTTTACCGCGTCCCGTCCACCCCCAGCTGCCTCATGGATCCGATGTCTGCGCCTGCGAGG  120
 P  I  T  R  T  Q  N  P  M  V  T  G  T  S  V  L  G  V  K  F              60
CCCATCACTCGGACCCAGAACCCCATGGTGACTGGTACATCCGTACTAGGGGTGAAGTCC  180
 D  C  G  V  V  I  A  A  D  M  L  G  S  Y  G  S  L  A  R  F              80
GACTGCGGAGTGGTGATTGCTGCAGACATGCTGGGCTCCTACGGCTCCCTGGCTCGTCTC  240
 R  I  I  S  R  I  M  R  V  N  D  S  T  M  L  G  A  S  G  D             100
CGCAATATCTCTCGTTTTATGCGACTCAACGACAGCACTATGTTAGGTGCTTCAGGAGAC  300
 Y  A  D  F  E  Y  L  K  E  V  L  G  E  M  V  I  D  E  E  L             120
TATGCTGATTTCCAGTATTTGAAGCAAGTTCTCGGCCAGATGGTGATTGATGAGGAGCTG  360
 F  G  D  G  H  S  Y  S  P  R  A  I  H  S  W  L  T  R  A  M             140
TTCGGAGATGGACATAGCTATAGTCCTAGAGCTATCCATTCATGGTTGACAAGGGCCATG3'420
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Figure 3 Sequence of the 5′ RN3 cDNA clone obtained by 5′ RACE

The nucleotide sequence and deduced amino acid sequence of the 5′ cDNA clone are shown. Nucleotides are numbered, with 1 being the first nucleotide of the putative initiation codon. The underlined sequence is the new data not obtained in [12].

the subunit [10,12]. RN3 was found to have a molecular mass of around 24 kDa and a pI value of about 5 (Figure 1).

Growing Rat-1 fibroblasts or human L-132 cells were labelled with [^{35}S]methionine for 1 h in order to investigate whether the N3 precursor protein could be detected. The N3 subunit was immunoprecipitated with the polyclonal anti-RN3 antibodies following dissociation of proteasomes in cell extracts by heating in the presence of SDS as described in the Materials and methods. The size of the N3 was determined by autoradiography of two-dimensional PAGE gels of immunoprecipitated material. Two radiolabelled protein bands were detected, one of 31 kDa and one of 24 kDa, both having a similar pI value (Figure 2). The absence of the precursor form in material immunoprecipitated with anti-proteasome antibodies suggests that the unprocessed N3 does not occur, even transiently, in mature proteasomes or proteasome subcomplexes (Figure 2).

The fact that the apparent size of the higher-molecular-mass immunoprecipitated protein was around 31 kDa, compared with a predicted size of 25.5 kDa from the published nucleotide sequence [12], suggested that the original cDNA was not a full-length clone. This possibility was investigated by 5′ RACE, using total RNA purified from Rat-1wt fibroblasts. The nucleotide sequence and deduced amino acid sequence of the clone obtained by RACE are shown in Figure 3. The additional 5′ sequence extends the open reading frame to 792 nucleotides, encoding a protein of 262 amino acids with a calculated molecular mass of 28.5 kDa.

The relationship between the two immunoprecipitated proteins was investigated in the L-132 cells by pulse–chase analysis, because the labelling of proteasomes was more effective in these cells than in the rat fibroblasts and there was no detectable difference in the size of the precursor or mature form in rat and human cells. Cells were labelled with [^{35}S]methionine for 15 min, followed by chases of various periods (indicated in Figure 4A). Samples containing equal numbers of counts from each immunoprecipitation were run on SDS/PAGE gels. As the time of chase increased, the 31 kDa band decreased, and this was accompanied by an increase in the 24 kDa band. The possibility that the 31 kDa band is processed during sample preparation can be ruled out, as samples taken at each time point were treated identically and immediately lysed in RIPA buffer. The more likely explanation for the decrease in the 31 kDa band is that it is post-translationally processed to a 24 kDa protein after its synthesis.

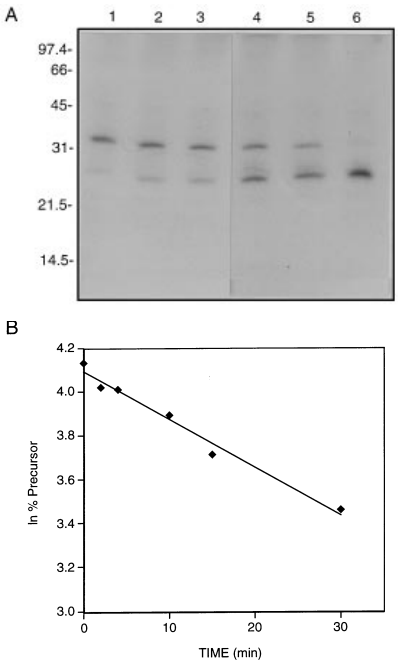

Figure 4 Half-life of processing of N3 in cultured L-132 cells

(**A**) Cells were labelled with [^{35}S]methionine for 15 min and then lysed after 0 min (lane 1), 2 min (lane 2), 4 min (lane 3), 10 min (lane 4), 15 min (lane 5), and 30 min (lane 6) chase times for immunoprecipitation of N3 with anti-RN3 antibodies. Equivalent amounts of immunoprecipitated radioactivity from each time point were loaded on to an SDS/PAGE gel and labelled proteins detected by autoradiography as detailed in the Materials and methods section. (**B**) Determination of the half-life for processing of the RN3 proprotein. Data in (**A**) were analysed by determination of the radioactivity in each radiolabelled band, as described in the Materials and methods section. The half-life of processing was determined using the equation $t_{\frac{1}{2}} = \ln 2/k$, where k is the gradient of the line.

The autoradiograph showed a decrease in the upper band with time of chase, and the half-life of this decrease was determined by quantifying the radioactivity in each band using a PhosphoImager. The amount of precursor at each time point was determined as a percentage of the total amount of N3 protein immunoprecipitated at that time point to correct for any differences in the total counts loaded on to the gel (Figure 4B). The half-life for processing in this experiment was determined to be 31 min, a value which is consistent with data obtained in other similar experiments.

DISCUSSION

Expression of the proteasome subunit RN3 protein in *E. coli* and subsequent raising of subunit-specific polyclonal antibodies has allowed identification of this subunit among the complex pattern of around 20 spots observed on two-dimensional PAGE gels of purified rat liver proteasomes [21]. Unlike the variable or non-essential beta-type proteasome subunits, such as LMP2 and LMP7, N3 is apparently present in all animal cell proteasomes and 26 S proteinase complexes. It is therefore not surprising that Western blots showed a distribution of RN3 in different rat tissues which paralleled that determined using anti-proteasome polyclonal antibodies [21]. A single mRNA transcript was found for RN3, whereas two different transcripts can be observed for subunits C5 [27,28] and LMP7 [15,29]. The position of the N3 protein on two-dimensional PAGE gels of rat and human proteasomes was consistent with calculated molecular mass

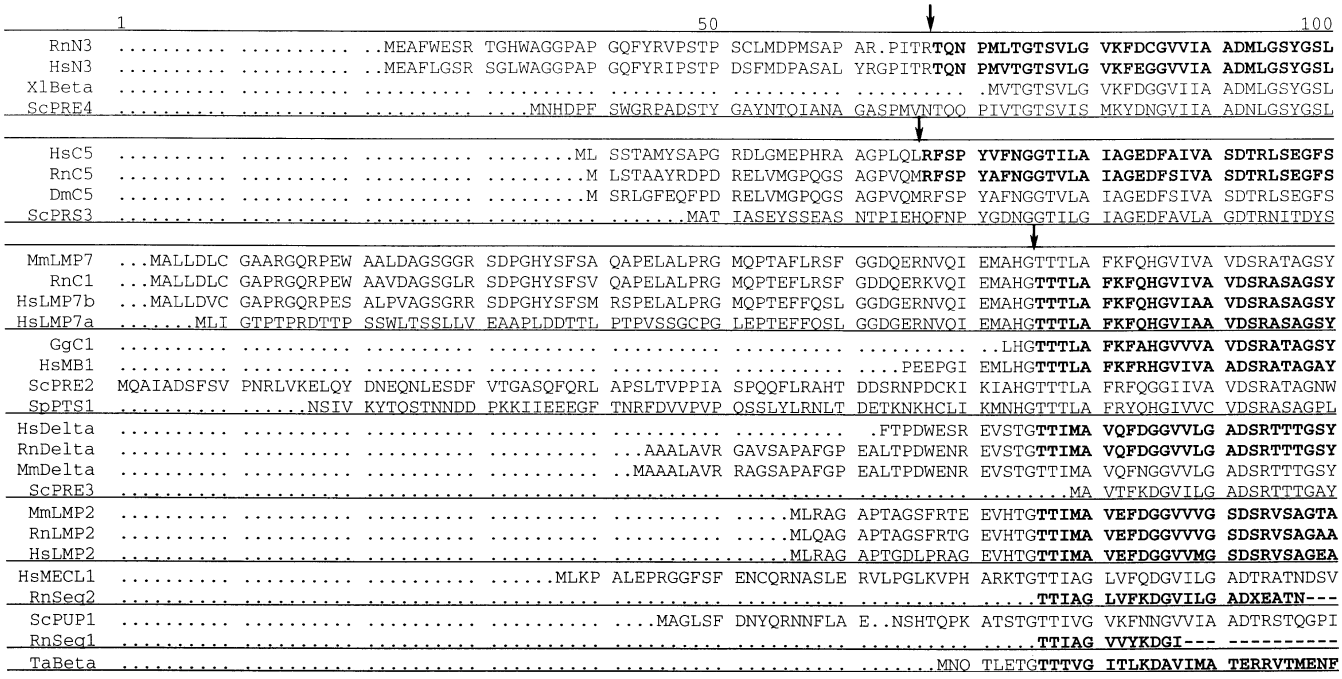

Figure 5 N-terminal processing of proteasome beta-type subunits

The Figure shows the alignment of N-terminal sequences of proteasome beta-type subunits. Abbreviations are as follows: Dm, *Drosophila melanogster* (fruitfly); Gg, *Gallus gallus* (hen); Hs, *Homo sapiens* (man); Mm, *Mus musculus* (mouse); Rn, *Rattus norvegicus* (rat); Sc, *Saccharomyces cerevisiae* (yeast); Sp, *Schizosaccharomyces pombe* (fission yeast); Ta, the protozoan *Thermoplasma acidophilium*; Xl, *Xenopus laevis* (toad). Sequence data were taken from the following sources; DmC5 [40]; GgC1 (EMBL database accession no. X57210); HsC5 [41]; HsN3 [42]; HsLMP7a and HsLMP7b [15]; HsMB1 [43]; HsDelta [44]; HsLMP2 [45]; HsMECL1 [46]; MmLMP7 [47]; MmLMP2 [9]; MmDelta [49]; RnC5 [27]; RnC1 [50]; RnDelta, RnLMP2 [51]; RnSeq1 and RnSeq2 [10]; ScPRE4 [13]; ScPRS3 [52]; ScPRE2 [53]; SpPTS1 (EMBL database accession no. D13094); ScPRE3 [38]; ScPUP1 [54]; TaBeta [55]. The additional N-terminal protein sequence for RnDelta was obtained by translation of the 5′ nucleotide sequence of the cDNA in the database. Species homologues are grouped together. Known N-terminal sequences of mature subunits are indicated in **bold**. The arrows (↓) indicate the proposed site of cleavage.

(24.5 kDa) and pI (5.63) values and with the recently reported position of the human placenta proteasome N3 subunit [30].

[^{35}S]Methionine labelling of cultured cells followed by immunoprecipitation with anti-RN3 antibodies and two-dimensional PAGE showed two labelled polypeptides. One of these corresponded to N-terminally processed N3 [10]; the other was likely to be the newly synthesized N3 proprotein. However, the size of the latter protein was higher than expected on the basis of previously reported cDNA sequences [12,31]. The full sequence of RN3, the remainder of which was obtained by 5′ RACE, gave a predicted total molecular mass of 28.5 kDa, a value which is close to that determined experimentally.

Alignment of the amino acid sequences of rat and human mature N3 subunits shows 95% identity. The sequence of the propeptide, which contains six proline residues in a total of 44 amino acid residues, is less highly conserved (70% identity). Although the beta subunit from *Xenopus* [32] is closely related to RN3 [12], it appears to have no 'pro' region. However, it is possible that this was not a full-length clone, since it was obtained by chance while screening a library of expressed genes in the intermediate pituitary glands of *Xenopus laevis*. Comparison of processed RN3 with the yeast homologue PRE4 shows 40% identity at the amino acid level. There is no data available yet concerning the processing sites in yeast proteasome subunits [7]. However, it seems likely the processing will occur at the equivalent site, since the sequence around the cleavage site is highly conserved. Interestingly the putative propeptide is not conserved, being much shorter (only 12 amino acids) in yeast.

The fact that none of the precursor form of RN3 could be detected in purified rat liver proteasomes suggests either that this bond is very susceptible to proteolysis during the purification, or that processing occurs during the assembly of active proteasomes. The results of Western-blot analysis of freshly prepared tissue extracts as well as those of [^{35}S]methionine labelling of cultured cells support the latter interpretation. Moreover, the processing occurs very rapidly, with an estimated half-life of 31 min. Our difficulty in detecting the unprocessed form of N3 using long labelling times simply reflects the short half-life of processing compared with that of protein turnover. The half-life of proteasomes in rat liver is 8–9 days [33], and in HeLa cells it is approx. 5 days [34].

N-terminal pro regions of proteinases often play a role in the inhibition of proteinase activity, but in other proteins they can play a role in targeting proteins to particular subcellular compartments or be required for correct protein folding [35]. Studies with the *Thermoplasma* proteasome, the two subunits of which have conveniently been expressed in *E. coli*, have shown that processing of the beta subunits accompanies assembly, but that beta subunits expressed alone without their eight-amino-acid propeptide can form active complexes [10]. The N-terminal threonine residue of the processed *Thermoplasma* beta subunit is believed to be the single catalytic residue [36]. In the case of proteasomes it is therefore likely that the function of the propeptide may be to maintain the subunits in an inactive form until they are properly assembled rather than to promote correct folding. As in the case of other proteinases, the new N-terminal residue has been found to be involved in the catalytic mechanism.

Eukaryotic proteasomes are considerably more complex than

the simple structure of the *Thermoplasma* proteasome, and animal cell proteasomes have at least seven different types of beta subunits and at least five distinct types of proteolytic activity [17]. Many of the animal proteasome beta subunits are cleaved at [(H/T)G↓TT(I/L)(L/M)], which is the equivalent site to that found in the *Thermoplasma* beta subunit (Figure 5). However, C5 and N3 are cleaved at [Q(L/M)↓RFSP] and (TR↓TQNP) respectively (Figure 5), suggesting that there is a different mechanism of cleavage at these sites and that a different type of catalytic activity is involved. Two of the animal proteasome beta subunits, namely C7 and C10, are N-terminally blocked. Our labelling studies have implicated RC7 in both the chymotrypsin-like and trypsin-like activities of rat liver proteasomes [37].

The yeast homologue of N3, the Pre4 subunit, has been implicated in the peptidylglutamyl-peptide hydrolase activity of the proteasome [13], along with another subunit, Pre3 [38], because yeast strains mutant in either of these subunit genes are defective in this type of activity. However, the relationship between the yeast peptidylglutamyl-peptide hydrolase activity and the two similar activities (LLE1, LLE2 [39]) of animal cell proteasomes has not been established.

It is noteworthy that the half-life of processing of N3 is much shorter than the value of 2 h estimated for the non-essential MHC-encoded subunit LMP7 [15], and this may have important implications for the mechanism of proteasome assembly. It has been suggested that processing of LMP2 and LMP7 occurs in proteasome precursor complexes [16], but we have found no evidence that the unprocessed form of N3 occurs in such complexes, suggesting that the mechanism of its processing and incorporation into proteasomes may differ from that of other beta subunits. The longer propeptides of some animal proteasome beta subunits compared with that of the *Thermoplasma* beta subunit suggests that proteasome subunit propeptides may also play a role in the organization of the complex.

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