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Isolation and characterization of the MHC linked β-type proteasome subunit MC13 cDNA

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We have cloned and analysed the second mouse MHC-linked proteasome subunit, designated MC13, which appears to be homologous to the human RING10 proteasome protein. The isolated cDNA has an ORF encoding a protein of 276 amino acids with a molecular weight of ca. 30 kDa. Sequence alignment reveals that the subunit MC13 and several other mammalian proteasome subunits are encoded by a second proteasome gene family. This second gene family encodes subunits of the β-type, reveals striking sequence similarities with the β-subunit of archaebacterial proteasomes and is related to, but distinct from, the genes encoding the so-called α-type subunits.

1. INTRODUCTION

The proteasome is a highly conserved intracellular 700 kDa multi-subunit proteinase complex which possesses several proteolytically active sites with trypsin-like, chymotrypsin-like and glutamyl hydrogen bond hydrolyzing activities (for review see [1–3]). Due to its biochemical properties the proteasome appears to be a candidate key enzyme of various non-lysosomal pathways of intracellular protein metabolism. More specifically, proteasomes are thought to play a role in ontogenetic regulatory mechanisms [4,5] and in ubiquitin-dependent proteolysis [6,7]. Recent reports have presented evidence that proteasomes are probably the proteolytic enzymes responsible for the generation of peptides presented by MHC-class I molecules [8–11]. Furthermore, two genes encoding proteasome subunits, i.e. RING10 and RING12, were identified within the human MHC II region while only one mouse MHC-linked proteasome subunit, i.e. LMP2, has been reported. Here we report the cloning of the cDNA of a second MHC encoded mouse proteasome subunit, which was designated MC13 following the nomenclature of Tanaka for mammalian proteasome subunits.

2. MATERIALS AND METHODS

2.1. *Screening procedure*

Four different oligonucleotides derived from the human RING10 cDNA sequence were used to screen a BALB/c B cell lymphoma A20 cDNA-library (Stratagene) using the method of Wood et al. [12]. After hybridization nitrocellulose filters were rinsed 3 times with 6 × NaCl/Cit at 4°C, washed with 3 M Me_4NCl for 10 min at 37°C and 30 min with 3 M Me_4NCl at 50°C. Filters were exposed overnight using a Kodak X-ray film.

The following primers were used for library screening (derivatives from the human RING10 cDNA-sequence) RING10/1,5'-ATGGCCCATGGCACCACCACGCTCGCCTT-3'(29 nucleotides); RING 10/2, 5'-GAGATTAACCCTTACCTGCTTGGCACCATG-3'(30 nucleotides); RING 10/3,5'-TGGGATAAGAAGGGTCCTGGACTCTACTAC-3'(30 nucleotides); RING 10/4,5'-CTATGACCTTGGCCGCAGGGCTATTGC-3'(27 nucleotides). RING10/1 plus RING10/2 probes were pooled and used for hybridisation of the first set of nitrocellulose filters. Pooled RING10/3 and RING10/4 probes were used for hybridisation of the replica nitrocellulose filters.

2.2. *Large-scale DNA preparation of MC13 and DNA sequencing*

Since the cDNA library A20 was purchased as λ-ZAP vector library. Bluescript plasmid containing the MC13 cDNA was easily obtained after cloning, following the excision method given by Stratagene for helper phages. Large-scale preparation of plasmid was performed using standard procedures. To sequence the entire MC13 cDNA, subclones in Bluescript vector from the whole cDNA were made after restriction enzyme digestion. In addition, MC13 cDNA was truncated stepwise by *Bal*31 treatment from both 5'- or 3'-end to obtain more appropriate fragments for sequencing. DNA sequencing was performed using the dideoxy method [14].

3. RESULTS AND DISCUSSION

To isolate the second MHC-linked mouse proteasome subunit we screened a mouse BALB/c B cell cDNA library using different oligonucleotides whose sequence was derived from the human RING10 cDNA. From 1×10^5 recombinants screened we obtained 12 positive cDNA clones. Of these, MC13 contained the largest cDNA insert and was therefore further analysed. The nucleotide sequence obtained and the predicted amino acid sequence are shown in Fig. 1. The isolated mouse cDNA MC13 possesses a DNA sequence identity with the homologous human RING10 cDNA of

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   1  GGCACGAGGCCAAGTGGTCATGGCGTTACTGGATCTGTGCGGTGCCGCTCGGGGACAGCGGCCCGAGTGGGCTGCCCTGG  80
   1                    M  A  L  L  D  L  C  G  A  A  R  G  Q  R  P  E  W  A  A  L  D  21

  81  ATGCGGGAAGCGGGGGTCGCTCGGACCCGGGACACTACAGTTTCTCCGCGCAAGCTCCGGAGCTCGCACTTCCCCGGGGA 160
  22    A  G  S  G  G  R  S  D  P  G  H  Y  S  F  S  A  Q  A  P  E  L  A  L  P  R  G   47

 161  ATGCAGCCCACCGCATTCCTGAGGTCCTTTGGTGGTGACCAGGAAAGGAATGTTCAAATTGAGATGGCCCACGGCACAAC 240
  48  M  Q  P  T  A  F  L  R  S  F  G  G  D  Q  E  R  N  V  Q  I  E  M  A  H  G  T  T   74

 241  CACACTCGCCTTCAAGTTCCAGCATGGCGTCATCGTGGCTGTGGACTCCAGGGCCACTGCAGGGAGTTACATTAGCTCCT 320
  75   T  L  A  F  K  F  Q  H  G  V  I  V  A  V  D  S  R  A  T  A  G  S  Y  I  S  S  L 101
                                                                αII
 321  TAAGGATGAACAAAGTGATCGAGATTAACCCTTACCTGCTTGGCACCATGTCTGGTTGTGCAGCCGACTGCCAGTACTGG 400
 102    R  M  N  K  V  I  E  I  N  P  Y  L  L  G  T  M  S  G  C  A  A  D  C  Q  Y  W  127

 401  GAGAGGCTGTTGGCCAAGGAGTGCAGGTTGTATTATCTTCGGAATGGGGAACGCATCTCCGTGTCTGCAGCATCCAAGCT 480
 128  E  R  L  L  A  K  E  C  R  L  Y  Y  L  R  N  G  E  R  I  S  V  S  A  A  S  K  L  154

 481  GCTTTCCAACATGATGCTGCAGTACCGGGGGATGGGCCTCTCCATGGGCAGCATGATCTGTGGCTGGGACAAGAAGGGAC 560
 155   L  S  N  M  M  L  Q  Y  R  G  M  G  L  S  M  G  S  M  I  C  G  W  D  K  K  G  P 181
                                                                                  βI
 561  CAGGACTTTACTACGTAGATGACAATGGGACTCGGCTCTCGGGACAGATGTTTTCCACTGGCAGCGGGAACACCTATGCC 640
 182    G  L  Y  Y  V  D  D  N  G  T  R  L  S  C  Q  M  F  S  T  G  S  G  N  T  Y  A  207

 641  TACGGGGGTGATGGACAGTGGTTACCGGCAGGACCTCAGTCCTGAAGAGGCCTACGACCTTGGCGGCAGAGCTATTGCTTA 720
 208  Y  G  V  M  D  S  G  Y  R  Q  D  L  S  P  E  E  A  Y  D  L  G  R  R  A  I  A  Y  234
                 βII
 721  TGCTACCCACAGAGACAACTATTCTGGAGGAGTCGTCAACATGTACCACATGAAGGAAGACGGTTGGGTGAAAGTGGAGA 800
 235   A  T  H  R  D  N  Y  S  G  G  V  V  N  M  Y  H  M  K  E  D  G  W  V  K  V  E  S 261

 801  GTTCCGATGTCAGTGACCTGCTGTACAAGTACGGAGAGGCCGCTCTGTGATGGCTGCTGGGCAGGCCTCCCCCAGCATTG 880
 262   S  D  V  S  D  L  L  Y  K  Y  G  E  A  A  L  *                               277

 881  GTGGCACTGGCTGGCAGACTCAGAGACCTGGGACTACTTCAGTCTTAGGAAAAAGAAGGGCTCAACCTGGGCTGGAGACA 960

 961  AAGCTCTGTTTACCCTCTCGGCCCCCGCACTCACAGATACCTTCTAAGTACAATAAAAGAAAAACGGTTAAAAAAAAAAA1040

1041  AAAAA  1045
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Fig. 1. Nucleotide sequence and the deduced amino acid sequence of the cDNA clone encoding the mouse proteasome MC13 subunit. The boxes depict the conserved PROS box domains.

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   1  MALLDLCGAARGQRPEWAALDAGSGGRSDPGHYSFSAQAPELALPRGMQPTAFLRSFGGD  60
      | | |          | ||     |       |      ||||| || |||||
   1     MLIGTPTPRDTTPSSWLTSSLLVEAAPLDDTTLPTPVSSGCPGLEPTEFFQSLGGD  56

  61  QERNVQIEMAHGTTTLAFKFQHGVIVAVDSRATAGSYISSLRMNKVIEINPYLLGTMSGC 120
      |||||||||||||||||||||||||| |||||| |||||| ||||||||||||||||||||
  57  GERNVQIEMAHGTTTLAFKFQHGVIAAVDSRASAGSYISALRVNKVIEINPYLLGTMSGC 116

 121  AADCQYWERLLAKECRLYYLRNGERISVSAASKLLSNMMLQYRGMGLSMGSMICGWDKKG 180
      ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
 117  AADCQYWERLLAKECRLYYLRNGERISVSAASKLLSNMMCQYRGMGLSMGSMICGWDKKG 176

 181  PGLYYVDDNGTRLSGQMFSTGSGNTYAYGVMDSGYRQDLSPEEAYDLGRRAIAYATHRDN 240
      |||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||||
 177  PGLYYVDEHGTRLSGNMFSTGSGNTYAYGVMDSGYRPNLSPEEAYDLGRRAIAYATHRDS 236

 241  YSGGVVNMYHMKEDGWVKVESSDVSDLLYKYGEAAL  276        MC13
      |||||||||||||||||||| ||||||  | ||
 237  YSGGVVNMYHMKEDGWVKVESTDVSDLLHQYREANQ  272        RING10
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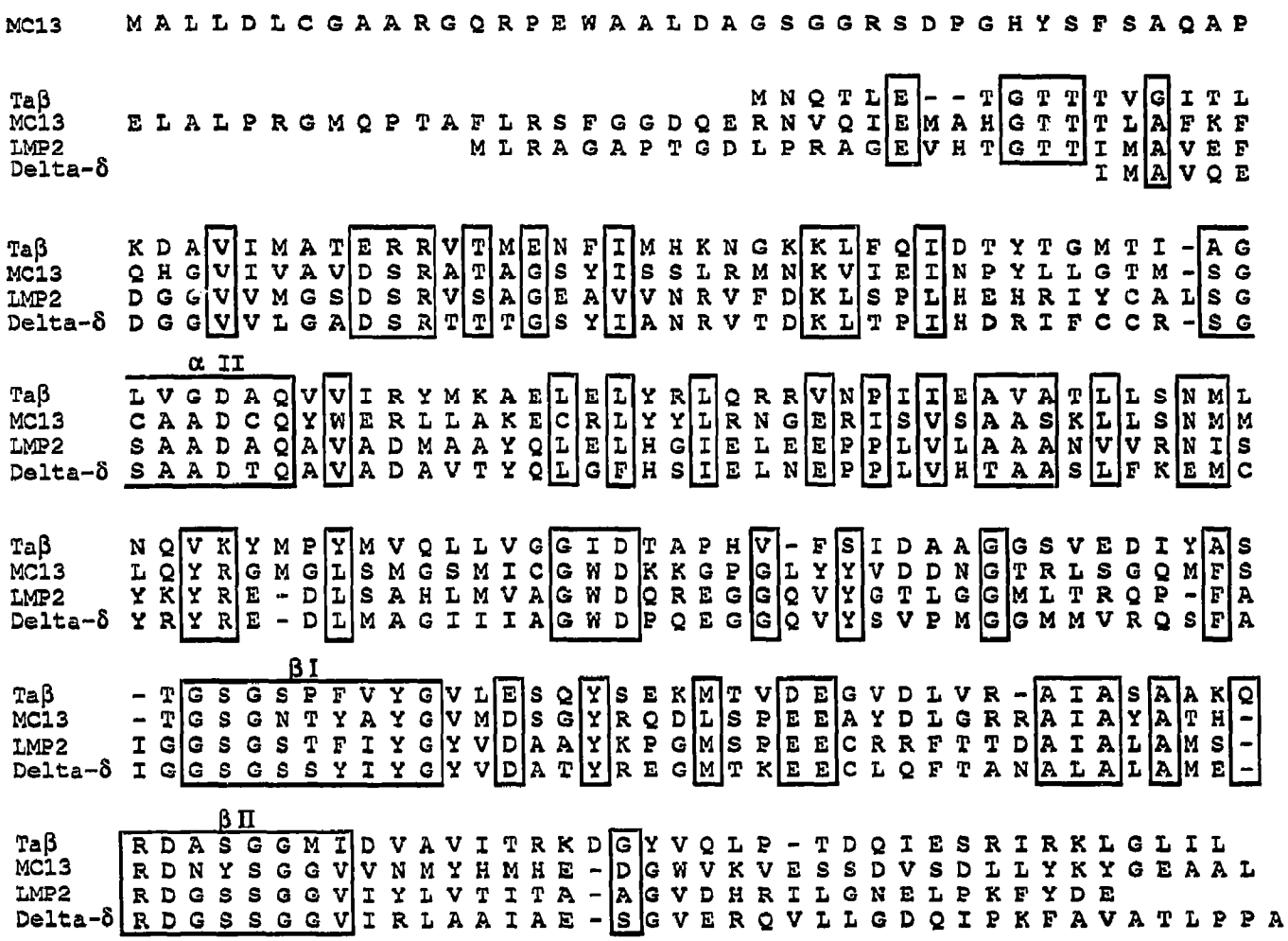

Fig. 3. Amino acid sequence alignment of four β-type proteasome subunits from archaebacteria (Taβ), mouse (MC13, LMP2) and human (Delta-δ) [21]. Sequences where at least three out of four amino acids are identical are boxed.

73.9%. MC13 has a length of 1045 nucleotides and contains a long open reading frame of 828 nucleotides encoding a protein of 276 amino acids with a calculated molecular weight of ca. 30 kDa and an IEP of 6.66. There are three ATG at position 20, 161 and 224 of the MC13 cDNA. Since the ATG at nucleotide 20 is located within a sequence related to the consensus sequence for initiation of translation [14] this first ATG most likely also represents the translational start site. The overall identity in amino acid sequence between the MC13 and the human RING10 protein is 60.8%. Amino acid sequence alignment of the two proteins, however, shown that the sequence homology in fact is divided into two parts (Fig. 2). Starting with the methionine residue at amino acid position 69, sequence identity between the two proteins rises to 91.4%. Nevertheless, even this slight divergence seems surprising since other homologous mammalian proteasome subunits possess a sequence identity of close to 100% [17]. The fact that starting with amino acid 69 the C-terminal two thirds of the protein also shows strong similarities to deduced sequences from other β-type proteasome subunits and sequences derived from N-terminal protein sequencing [15,16] might suggest that the ATG at nucleotide position 224 serves as a translational start codon. Although this possibility cannot be ruled out entirely, it is intriguing to see that both the amino acid sequence identity and the conservation between the two proteins goes beyond this methionine, arguing also in favour of a translational start upstream of this position.

←

Fig. 2. The amino acid sequence similarities between the human RING10 proteasome subunit and the mouse MC13 proteasome subunit are shown. The domains forming a putative active site are underlined.

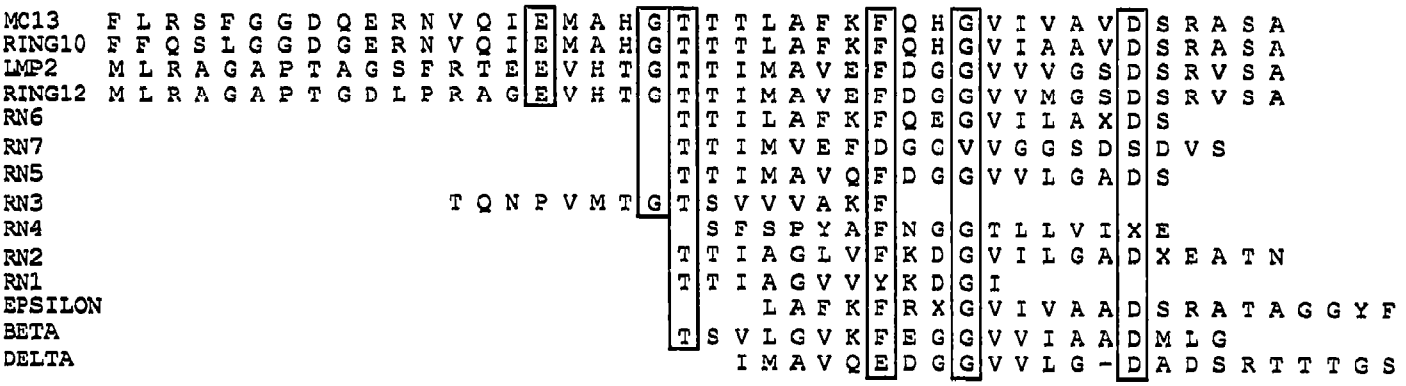

Fig. 4. Amino acid sequence alignment of N-terminal amino acid sequences deduced from cloned DNAs (MC13, RING10, LMP2, RING12) and amino acid sequences derived from N-terminal protein sequencing of isolated proteasome subunits. The most conserved amino acids are boxed.

3.1. *MC13 is a β type proteasome subunit*

Based on the molecular analysis of the genes encoding the archaebacterial α and β subunits, it appears that these two genes have given rise to two different but related proteasome gene families encoding subunits which are similar to either the α- or β-type archaebacterial subunit [18,19]. Sequence alignment clearly identifies MC13 as well as the other MHC-linked subunit LMP2 as a member of the β-type proteasome subunits (Fig. 3). The β-type subunits differ from the α-type subunits in that they lack α-type PROS-boxes I and III [20]. Further, β-type subunits possess different subtypes of the α-type PROS-box II. In addition, the β-type subunits shown in Fig. 3 possess two conserved domains, i.e. PROS-boxes βI and βII which are not found in any of the α-type subunits and which only appear to be present in a subgroup of β-type subunits. In most cases N-terminal sequencing of unblocked proteasome β-type subunits reveals an N-terminal threonine residue [15,16]. Comparison of those data with the amino acid sequences shown in Fig. 4 shows that this threonine is preceded by additional – and between different subunits, varying numbers of – amino acid residues towards the N-terminius (Fig. 4). This is also true for the MC13 subunit described here, whereby the threonine in question at amino acid position 72 is preceded by a glycine residue. If, as one may conclude from protein sequencing data of mammalian and archaebacterial proteasome β-type subunits, the threonine residue is indeed the N-terminal amino acid of the β-type subunits which are incorporated into the proteasome, it has to be postulated that at least a subset of proteasome subunits undergoes post-translational processing. Thus there exists the likely possibility that proteasome enzyme subunits are synthesized as precursors and become processed for activation or incorporation into the multi-enzyme complex. MC13, like all other proteasome subunits identified so far lacks homologies to known proteinases. Interestingly, Glynne et al. [9] discussed the possibility that the human RING10 proteasome subunit possesses homology to a serine proteinase-type active site found in subtilisin. However, since MC13 as a homologous subunit possesses an asparagine instead of an histidine within the domain defining the active site (Fig. 2) the functional significance of this homology appears doubtful. Nevertheless, judging from the evolutionary distance the close homology of MC13 to the archaebacterial β subunit is striking and suggests a potentially similar function of the proteins within the enzyme complex. From circumstantial evidence it was suggested [19] that the β-type subunits are responsible for the proteolytic activity of the proteasome complex. In fact, a point mutation within a β-type subunit of yeast proteasomes was shown to affect the chymotrypsin-like activity of the complex [22]. Whether the MHC-linked proteasome subunits contribute to a similar type of proteolytic activity, and whether this can be correlated to the potential antigen processing activity of the proteasome, remains to be shown.

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