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MOLECULAR CLONING OF THE SMALLER SUBUNIT(P52) OF RAT LIVER MITOCHONDRIAL PROCESSING PROTEASE

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Summary: A cDNA encoding the smaller subunit (P52) of mitochondrial processing protease was isolated from a rat liver cDNA library, using cDNA fragment for yeast MAS1 as the probe. The deduced amino acid sequence is highly homologous to those of PEP from *Neurospora crassa* and MAS1 from *Saccharomyces cerevisiae*. After *in vitro* transcription and translation, the precursor peptide was imported into isolated rat liver mitochondria and processed to its mature form.

Most mitochondrial proteins are synthesized on cytoplasmic ribosomes as larger precursors with amino-terminal extension peptides of 20 to 60 amino acid residues. After synthesis, precursors are imported into mitochondria by a multi-step process, including unfolding, specific binding to mitochondrial surface, energy-dependent translocation across the membranes, and removal of the extension peptides from the precursors by matrix-located protease, i.e. mitochondrial processing protease (1, 2). The cleavage of the extension peptide is not coupled to the translocation of the precursors across the membranes (3, 4), but probably required for folding and assembly of the newly imported peptides into the functional forms.

Mitochondrial processing proteases have been purified from *Neurospora crassa* (5), *Saccharomyces serevisiae* (6), and rat liver (7, 8). The purified proteases require a metal for the activity and form heterodimeric complexes. The rat enzyme consists of two protein components of 55 kD (P55) and 52 kD (P52) (7). Primary structures of these subunits, except rat P52, have been revealed (5, 9-13). Larger subunits of the processing protease, i.e. rat liver P55, *Neurospora* MPP and yeast MAS2, are highly homologous among them (9, 11-13).

To elucidate the role of each subunit and the molecular mechanism of cleavage of extension peptides, we attempted to isolate rat P52 cDNA. In this

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communication, we describe the primary structure and some properties of rat P52.

MATERIALS AND METHODS

cDNA Cloning and Sequencing of P52 of Rat Liver Mitochondrial Processing Protease: A λgt11 rat liver cDNA library was screened for P52, using a cDNA fragment for yeast MAS1, which is a homologue to P52, as the probe (14). The MAS1 cDNA fragment was prepared by polymerase chain reaction from λgt11 yeast cDNA library (14), using sense (base number 862 to 885 of MAS1 gene (10)) and antisense (1384 to 1407) oligonucleotides as the primers. The sequence selected above is a highly conserved portion between *Neurospora* PEP and yeast MAS1. The positive cDNA clone with the longest length was sequenced (15).

In Vitro Import of Precursors of P52 and P55 into Rat Liver Mitochondria: After substitution of methionine codon for the first alanine codon, the P52 cDNA was introduced into Bluescript SK^+ vector and transcribed *in vitro* under the control of the T7 promoter, as previously described (4). The mRNA was translated to get [^{35}S]-labeled precursor in rabbit reticulocyte lysate (16). P55 precursor was also prepared as previously described (4). Import of the precursors into isolated mitochondria was performed as described (17). After the import reaction, the mitochondria were further incubated with or without proteinase K for 30 min at 0°C. The import and processing was analyzed by SDS-PAGE and fluorography. Rat liver mitochondria were prepared as described (7). The cDNA for P55 was cloned according to Kleiber *et al.* (9).

In Vitro Processing of Precursors of P52 and P55 by Rat Liver Mitochondrial Matrix Fraction: Precursors of P52 and P55 were incubated with the matrix fraction of rat liver mitochondria. The processing of the precursors was analyzed as described (7).

RESULTS AND DISCUSSION

A cDNA clone encoding smaller subunit of rat liver mitochondrial processing protease, P52, was isolated from rat liver cDNA library, using a cDNA fragment (about 500 bp) for yeast MAS1 (10) as the probe. Figure 1 shows the nucleotide and deduced amino acid sequences. The cDNA is 1570 nucleotides long and encodes 487 amino acids. Although it does not contain the initial ATG codon, amino-terminal portion of the protein has the characteristic feature common to the extension peptides of mitochondrial protein precursors; presence of several basic amino acid residues intervened with uncharged residues (1, 2).

The amino acid sequence of rat P52 shows 49% and 46% homology to those of PEP from *Neurospora crassa* (5) and MAS1 from yeast, *Saccharomyces cerevisiae* (10), respectively (Fig. 2). Striking homology among them was found in the amino-terminal portion, where acidic residues are rich. The sequence of P52 also shows 30% homology to that of P55 (data not shown). Similarity between the larger and smaller subunits was also observed in yeast and *Neurospora* enzymes (11). Rat P52 has a sequence of HFLEH (underlined

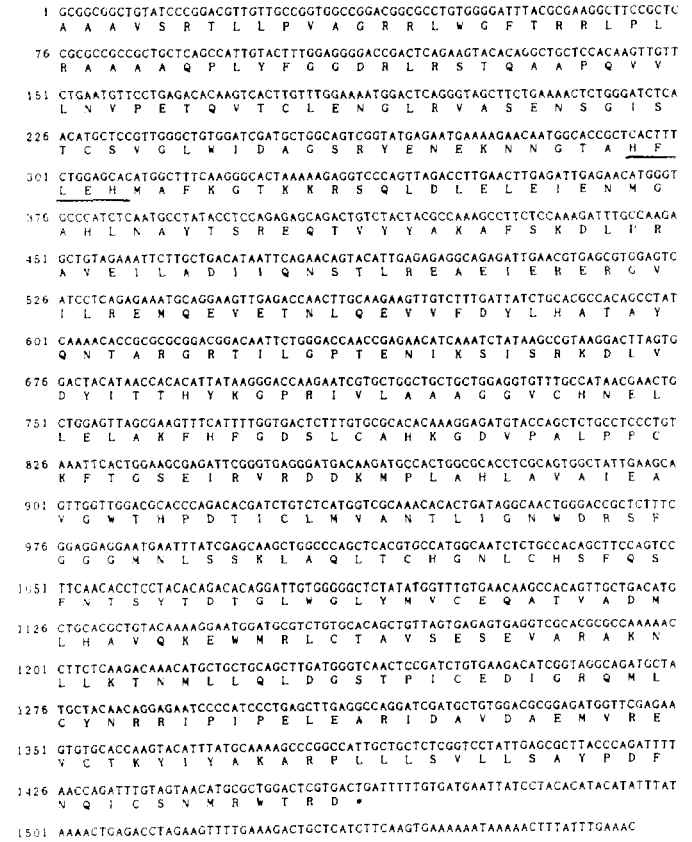

Fig. 1. Nucleotide sequence of the cDNA for P52 subunit of rat mithochondrial processing protease and its deduced amino acid sequence. Amino acids are denoted by single-letter code. Stop codon is indicated by asterisk. A putative metal binding motif is underlined.

in Fig. 1), which is the inverted sequence of the metal binding motif (HEXXH) common to metalloendoproteases (18, 19). The inverted motif , HXXEH, was found in *E. coli* protease III and confirmed to participate in binding of zinc (20). This motif is well consereved among the smaller subunits of the proteases from three organisms (Fig. 2). On the other hand, the larger subunits have none of these motifs, although they has been considered to be responsible for the proteolytic activity (5). Hawlitschek *et al.* reported that, in *Neurospora* enzyme, the larger subunit (MPP) had a very low proteolytic activity and this activity was enhanced by the addition of the smaller subunit (PEP) which has no apparent activity (5). These inconsistent data could be interpreted as follows; (i) the smaller subunit has a metal which binds to the HXXEH sequence, and participates in the catalytic activity, (ii) the larger subunit is a new type of metalloprotease which has another metal binding structure. (iii) The processing protease is not a metalloprotease and a metal is involved in other functions instead of formation of a catalytic site. Further studies will be required to test which is the case.

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N.C.PEP                    MASRRLALNLAQGVKARAGGVINPFRRGLATPHSGTGI--KTQTTTLKNGLTVASQYSPYAQTSTVGMWIDAGSR
RAT P52          AAAVSRTLLPVAGRRLWGFTRRLPLRAAAAQPLYFGG--DRLRSTQAAPQVVLNV-PETQVTCLENGLRVASENSGIS-TCSVGLWIDAGSR
YEAST MAS1                                                MFSRTASKFRNTRRLLSTISSQIPGTRTSKLPNGLTIATEYIPKTSSATVGIFVDAGSR

AETDETNGTAHFLEHLAFKGTTKRTQQQLELEIENMGAHLNAYTSRENTVYFAKALNEDVPKCVDILQDILQNSKLEESAIERERDVILRESEEVEKQLEEVVFDHLHAT
YENEKNNGTAHFLEHMAFKGTKKRSQLDLELEIENMGAHLNAYTSREQTVYYAKAFSKDLPRAVEILADIIQNSTLREAEIERERGVILREMQEVETNLQEVVFDYLHAT
AENVKNNGTAHFLEHLAFKGTQNRSQQGIELEIENIGSHLNAYTSRENTVYYAKSLQEDIPKAVDILSDILTKSVLDNSAIERERDVIIRESEEVDKMYDEVVFDHLHEI

AYQHQPLGRTILGPRENIRDITRTELVNYIKNNYTADRMVLVGAGGVPHEQLVEMADKYFSKLPATAPVSSASILSKKKPDFIGSDIRIRDDTIPTANIAIAVEGVSWSD
AYQNTARGRTILGPTENIKSISRKDLVDYITTHYKGPRIVLAAAGGVCHNELLELAKFHFGD---SLCAHKGDVPALPPCKFTGSEIRVRDDKMPLAHLAVAIEAVGWTH
TYKDQPLGRTILGPIKNIKSITRTDLKDYITKNYKGDRMVLAGAGAVDHEKLVQYAQKYFGHVPKSESPVPLGSPRGPLPVFCRGERFIKENTLPTTHIAIALEGVSWSA

DDYFTGLVTQAIVGNYDKALGNAPHQGSKLSGF-VHKHDLATSFMSFSTSYSDTGLWGIYLVTD-KLDRVDDLVHFSLREWTRL-CSNVSDREVERAKAQLKASILLSLD
PDTICLMVANTLIGNWDRSFGGGMNLSSKLAQLTCH-GNLCHSFQSFNTSYTDTGLWGLYMV--CEQATVADMLHAVQKEWMRL-CTAVSESEVARAKNLLKTNMLLQLD
PDYFVALATQAIVGNWDRAIGTGTNSPSPLAVAASQNGSLANSYMSFSTSYADSGLWGMYIVTDSNEHNVQLIVNEILKEWKRIKSGKISDAEVNRAKAQLKAALLLSLD

GTTAVAEDIGRQLVTTGRRMSPAEIERIIDAVSAKDVMDFANKKIWDQDIAISAVGSIEGLFDYARIRGDMSRNAF
GSTPICEDIGRQMLCYNRRIPIPELEARIDAVDAEMVREVCTKYIYAKARPLLLSVLLSAYPDFNQICSNMRWTRD
GSTAIVEDIGRQVVTTGKRLSPGGVFEQVDKITKDDIIMWANYRLQNKPVSMVALGNTSTVPNVSYIEEKLNQ
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Fig. 2. Alignment of smaller subunits of mitochondrial processing proteases, i.e. Neurospora (N. C.) PEP, rat liver P52, and yeast MAS1. Residues identical to those of rat homologue are shaded. Dashed lines indicate gaps to maximize identity. Alignment was performed using the computer program GENETIX.

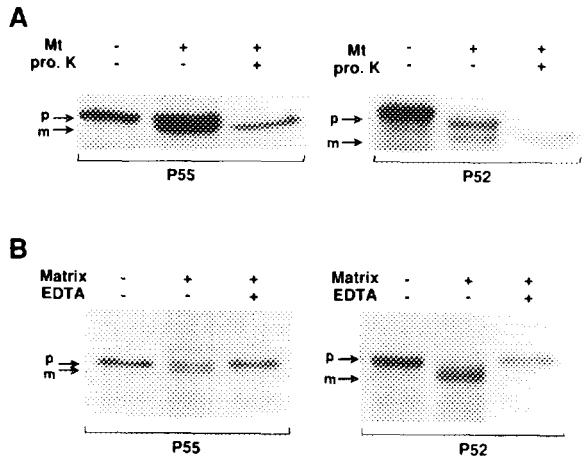

Fig. 3. A; Import of P55 and P52 precursors into rat liver mitochondria. Import was performed as described under "MATERIALS AND METHODS". After incubation of mitochondria (120µg protein) with P55 or P52 precursors synthesized in rabbit reticulocyte lysate, mitochondria were sedimented and subsequently treated with 0.5 µg of proteinase K for 30 min at 0°C.
***B**; In vitro processing of precursors.* Precursors synthesized *in vitro* were incubated with rat liver mitochondrial matrix fraction for 30 min at 30°C. **p**, precursor form; **m**, mature form; **Mt**, mitochondria; **Pro. K**, proteinase K.

When [^{35}S]-labeled precursors of P55 and P52 were synthesized in a reticulocyte lysate cell-free translation system and incubated with isolated rat liver mitochondria, they were imported into the mitochondria and processed to the mature peptides (Fig. 3A). Since several amino acid residues at the amino-terminal end of the extension peptides is indispensable for import of the precursors into mitochondria (17, 21), cDNA for P52 isolated here would be nearly full-length. The processing of the precursors, which was sensitive to EDTA, were also observed when precursors were incubated with the matrix fraction of rat liver mitochondria (Fig. 3B).

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