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In vitro processing of the precursor to human mitochondrial cytochrome c_1

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The processing of the precursor to human cytochrome c_1 has been studied using a transcript of a previously obtained human cytochrome c_1 cDNA. The transcript was prepared with SP64 plasmid containing the cDNA as a template, and was translated in a reticulocyte lysate cell-free translation system. The molecular mass of a major translation product was estimated to be 34 kDa. The product was shown to be imported into mitochondria, being converted into a mature-sized polypeptide (31.5 kDa), which was resistant to trypsin treatment. Thus, the former was considered to represent the precursor to the latter. The import required the energized state of the inner membrane. It was also demonstrated that a matrix proteinase cleaved the precursor to a form which was not distinguishable in size from the mature-sized one.

Introduction

Import of cytoplasmically-translated precursors into mitochondria has been extensively studied in lower eukaryotic cells, and it has been shown that most of the precursors have an N-terminal extension which functions as an import signal and is cleaved off in the process of import [1]. In higher eukaryotes, transport of mitochondrial matrix enzymes, such as malate dehydrogenase [2,3] and ornithine transcarbamylase [3,4], have recently been examined in detail. However, only little information is available on the processing of less abundant mitochondrial inner membrane proteins in mammalian cells.

Cytochrome c_1 is one of the mitochondrial electron-carrier proteins which is located on the inner membrane, protruding into the intermembrane space. We have recently isolated a cDNA encoding a putative precursor to human cytochrome c_1 from a human fibroblast cDNA library [5]. The nucleotide sequence of the cDNA showed that it contained an open reading frame coding for 325 amino acids comprising a protein with a molecular mass of 35 375 Da. On the other hand, Kolarov and Nelson [6] previously reported that the molecular mass of the precursor to rat cytochrome c_1 was 32 kDa. Thus, there is a large difference between the size determined for the rat precursor by their study and that deduced for the human precursor from the analysis of the cDNA. In the present study, we have examined the size of the precursor to human cytochrome c_1 by an in vitro transcription/translation experiment of the cDNA, and, at the same time, have studied the processing of it.

Materials and Methods

Materials

Trypsin and soybean trypsin inhibitor were obtained from Boehringer Manheim, the RNA cap structure analog GpppG from New England Biolabs and [^{35}S] methionine from New England Nuclear. Cytochrome c_1 [7] was purified from beef heart mitochondria as described. Antibody against the cytochrome c_1 was raised in a rabbit by intradermal injection of 0.5 mg of the protein in 1 ml of 0.9% NaCl, which had been emulsified with 1 ml of Freund's complete adjuvant. Booster injection of the same mixture was administered 3, 5,

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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and 7 weeks later. One week after the last injection, blood was withdrawn from the carotid artery.

Preparation of mRNA

The cloning of a cDNA encoding human cytochrome c_1 from a human fibroblast cDNA library in an Okayama-Berg vector, pcD [8], was described previously [5]. A fragment containing the cDNA was obtained by digestion with *Bam*HI and inserted into the *Bam*HI site of SP64 plasmid for subcloning. After linealization of the recombinant plasmid with *Pst*I, the 5′-flanking portion of the cDNA insert was deleted from the *Pst*I site with *Bal*31. The deleted cDNA was isolated after digestion at the *Eco*RI site in the polylinker region of SP64 plasmid and ligated with *Sma*I/*Eco*RI-cut SP64 plasmid. The resulting cDNA started from eight nucleotides upstream from the initiation codon as revealed by nucleotide sequence analysis. The recombinant plasmid DNA (1.5 μg) was linearized with *Eco*RI for transcription from SP6 promoter. Capped mRNA was synthesized using an SP6/T7 transcription kit (Boehringer Manheim) according to the manual supplied by the manufacturer with modifications. GpppG was added at 0.5 mM, and the concentration of GTP was 0.05 mM. The template DNA was removed by treatment with DNaseI, and the transcript was purified by the spun-column procedure [9] and dissolved in 30 μl of water.

Protein synthesis

Cell-free translation of the transcript was carried out using a rabbit reticulocyte lysate translation kit (New England Nuclear) according to the manual supplied by the manufacturer, except that the potassium acetate concentration was 80 mM. Incubation was carried out for 1 h at 30°C in 200 μl of an in vitro translation mixture containing the transcript (approx. 120 μg) and 400 μCi of [^{35}S]methionine.

Isolation and subfractionation of mitochondria

Intact mitochondria were isolated from rat liver by the method of Schnaitman and Greenawalt [10]. The mitochondria were subfractionated by the digitonin procedure with modifications [10,11]. Mitoplasts were suspended in 20 mM potassium Hepes (pH 7.4)/1 mM dithiothreitol at a concentration of 38 mg protein/ml, and disrupted by sonication (Branson Sonifier) with a microtip at maximum power for 1 min. The solution was centrifuged at 200000 × *g* for 30 min with a Himac centrifuge CP100H (Hitachi). The matrix fraction was stored in small aliquots at −80°C. The proteins of the mitochondria and the matrix fraction were determined with BCA protein assay reagent (Pierce). The intactness of the obtained mitochondria was checked by measurement of respiratory control ratio [12].

Processing of proteins

The translation mixture (10 μl) was mixed with 20 μl of a suspension of freshly isolated rat liver mitochondria (13 mg protein/ml) and incubated at 30°C for various periods of time. The mitochondria were separated from the supernatant and washed twice with 220 mM mannitol/70 mM sucrose/2 mM potassium Hepes (pH 7.4). When indicated, the mitochondrial pellets were treated with trypsin (40 μg/ml) for 30 min at 4°C and then supplemented with soybean trypsin inhibitor (125 μg/ml) or CCCP was added to the mitochondrial suspension prior to addition of the translation mixture. To test the translation product for processing with the matrix fraction, 5 μl of the translation mixture and 10 μl of the matrix fraction (4.4 mg/ml) were mixed and incubated in the presence and absence of 5 mM EDTA at 30°C.

Analytical procedures

Immunoprecipitation of translation product [13] and SDS-PAGE [14] were performed as described previously. Fluorography of gels was carried out using EN^{3}HANCE (New England Nuclear).

Results

Identification of the precursor to cytochrome c_1

To investigate into the relationship between the precursor and the mature form of mammalian cytochrome c_1, an in vitro transcription/translation experiment was done using, as a template, SP64 plasmid containing human cytochrome c_1 cDNA. The major polypeptide produced in the presence of [^{35}S]methionine gave a band at the position of a molecular mass of 34 kDa on SDS-PAGE followed by fluorography (Fig. 1, lane 2). This molecular mass agrees, within the limit of precision of the method used, with that calculated for human cytochrome c_1 from the gene data (35375 Da) [5] and is larger by approximately 2.5 kDa than the mature form of bovine cytochrome c_1 (Fig. 1, lane 1), which has almost the same molecular mass as its human counterpart [14]. Moreover, it was shown that the product formed in the transcription/translation experiment is immunoprecipitated by antibody directed against bovine cytochrome c_1 (Fig. 1, lane 3). Therefore, the product is considered to represent the precursor to human cytochrome c_1.

Transport into mitochondria

To examine whether the 34-kDa polypeptide is imported into mitochondria, we used a heterologous system in which the human polypeptide was tested for import into rat liver mitochondria. This is because intact human mitochondria cannot be easily prepared. When the polypeptide was incubated with rat liver mitochondria and was directly analyzed, two bands

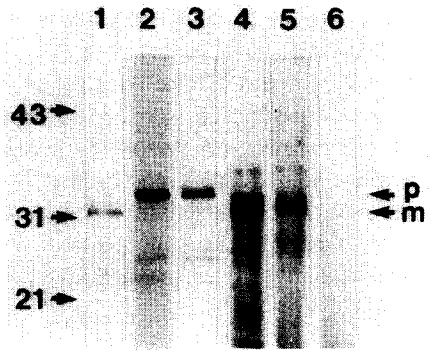

Fig. 1. The analysis of the precursor to human cytochrome c_1. A transcript of human cytochrome c_1 cDNA was prepared with SP64 plasmid, and used as a message for translation in a reticulocyte lysate cell-free system. The translation product was analyzed by SDS-PAGE followed by fluorography. Lane 1, beef heart cytochrome c_1 (1 μg) was stained with Coomassie blue. Lane 2, the translation product (0.3 μl) was analyzed directly. Lane 3, the translation product (3 μl) was immunoprecipitated with anti-cytochrome c_1 antiserum. Lane 4, the translation product (10 μl) was incubated with rat liver mitochondria (26 μg of protein) for 20 min at 30 °C. The mitochondria were washed and analyzed directly. Lane 5, after incubation of the mitochondria in the experiment for lane 4, the mitochondria were treated with trypsin before analysis. Lane 6, the translation product (10 μl) was incubated with mitochondria in the presence of 20 μM CCCP and then the mitochondria were subjected to trypsin treatment. The molecular masses of standard proteins (Pharmacia) are shown in kDa on the left side. p, the precursor to cytochrome c_1; m, the mature form of cytochrome c_1.

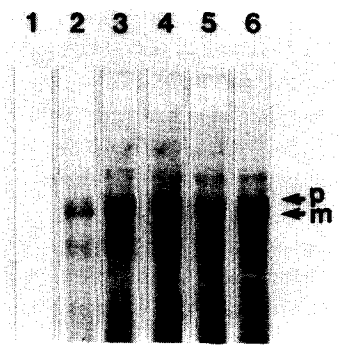

Fig. 2. The time course of import of the human cytochrome c_1 precursor into mitochondria. The translation product was incubated with rat liver mitochondria at 30°C for varying periods of time and aliquots of the mixture were removed. Lane 1, 0 time; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 40 min. The mitochondrial pellets (26 μg of protein) were analyzed after trypsin treatment. Lane 6, the mitochondria incubated with the translation product at 30°C for 20 min were analyzed without trypsin treatment. p, the precursor to cytochrome c_1; m, the mature form of cytochrome c_1.

were observed, one at the position of 34 kDa and the other at the position of 31.5 kDa (Fig. 1, lane 4). The electrophoretic mobility of the latter was the same as that of bovine cytochrome c_1; therefore, the 31.5-kDa polypeptide is thought to represent the mature form of human cytochrome c_1. The 31.5-kDa polypeptide became resistant to trypsin treatment after import into the intact mitochondria, but the 34-kDa polypeptide disappeared after trypsin treatment (Fig. 1, lane 5). This finding indicates that the 34-kDa polypeptide only attached to the surface of mitochondria and was not imported into mitochondria. CCCP, an uncoupler for mitochondrial oxidative phosphorylation, completely blocked the incorporation of the 34-kDa polypeptide (Fig. 1, lane 6), indicating that the import of the translation product of cytochrome c_1 precursor requires the energized state of mitochondrial inner membrane as other mitochondrial proteins do. In the experiment for the time course of import of the precursor, the amount of the mature protein detected reached the maximum at 20 min and then gradually decreased (Fig. 2). No intermediate form was detected during the course of the reaction.

Processing by matrix protease

When the traslation product formed using the transcript from cytochrome c_1 cDNA was incubated with the mitochondrial matrix fraction, it was converted into a polypeptide whose size could not be discerned from the size of the mature form by SDS-PAGE (Fig. 3A). When incubation was carried out in the presence of 5 mM EDTA, the formation of the mature-sized polypeptide was completely inhibited (Fig. 3B). These results indicate that a matrix proteinase, which is inhibitable by the metal chelator, catalyzes the cleavage of the human cytochrome c_1 precursor into the mature form.

Discussion

Sequence analysis of the cDNA for human cytochrome c_1 indicated that there were three methionine codons upstream from the starting point of the mature protein [5]. Among these, the first methionine codon is

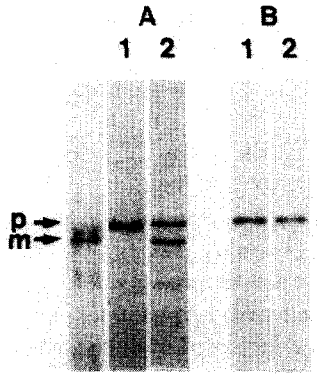

Fig. 3. The cleavage of the cytochrome c_1 precursor with matrix protease. (A) The translation product was incubated with the rat matrix fraction at 30°C for 30 min. Lane 1, 0 time; lane 2, 30 min. The leftmost lane, the translation product (10 μl) was incubated with mitochondria (26 μg) and the mitochondrial pellet was subject to trypsin treatment. (B) The incubation of the translation product with the matrix fraction was carried out in the presence of 5 mM EDTA. Lane 1, 0 time; lane 2, 30 min. p, the precursor to cytochrome c_1; m, the mature form of cytochrome c_1.

thought to function as the initiation codon of the cytochrome c_1 mRNA, because the sequence surrounding it has the feature of the initiation consensus sequence described by Kozak [15], but the others do not. The open reading frame starting from the first methionine codon encodes a protein of 325 amino acids with a molecular mass of 35 375 Da [5].

The molecular mass of the polypeptide produced by the in vitro transcription/translation experiment using the human cytochrome c_1 cDNA was estimated to be 34 kDa, which agrees reasonably well with the above predicated value. Kolarov and Nelson [6] previously reported that the molecular mass of the cytochrome c_1 precursor of rat hepatoma cells was 32 kDa. This value seems to be underestimated, since the size of both human and rat cytochrome c_1 precursors are comparable to each other as deduced from sequence analysis of rat cytochrome c_1 cDNA (unpublished data from this laboratory).

In our study, the molecular masses of mature cytochrome c_1 of both human and bovine were estimated to be 31.5 kDa. The molecular mass of bovine cytochrome c_1 estimated by SDS-PAGE has been reported to range from 29 kDa to 33 kDa [6,16,17]. Thus, the molecular mass to be estimated by SDS-PAGE tends to be considerably larger than the molecular mass (including the heme) of 27 924 Da [18] calculated from the amino acid sequence of bovine mature cytochrome c_1. Since the molecular mass (including the heme) of human mature cytochrome c_1 (28 132 Da) calculated from the deduced amino acid sequence is comparable to that of the bovine protein, the overestimation of the molecular mass of the mature form by SDS-PAGE may explain the estimated smaller difference in molecular mass (2.5 kDa), between the precursor and the mature form of human cytochrome c_1 than is expected from the theoretical values (7 243 Da).

It has been reported that in yeast, the processing of cytochrome c_1 proceeds in two steps with formation of an intermediate form [19] and that purified matrix processing protease cleaves the precursor to *Neurospora* cytochrome c_1 into an intermediate form [20]. Because cytochrome c_1 presequences of both yeast and human have a similar structural feature, viz., occurrence of a basic region at the N-terminus and a largely uncharged region at the C-terminus [21,22], occurrence of an intermediate form would be presumed for the processing of the human cytochrome c_1 precursor. However, no intermediate form was detected in our study of processing of the human cytochrome c_1 precursor. Kolarov and Nelson [6] were also unable to detect any intermediate form in the processing of the precursor to rat hepatoma cytochrome c_1. The intermediate form, if it appears in the processing of the human precursor, may not be resolved from the primary precursor or the mature form in the electrophoretic system used; or the intermediate form may be rapidly converted into the mature form. Alternatively, it is possible that the primary precursor is converted into the mature form without formation of any intermediate.

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