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An extremely acidic amino-terminal presequence of the precursor for the human mitochondrial hinge protein

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Mitochondrial hinge protein is a subunit of ubiquinol-cytochrome-*c* reductase in the respiratory chain and 'hinges' cytochrome *c* with cytochrome c_1. The protein is encoded in the nuclear genome, synthesized in the cytosol and then imported into the mitochondria. The cDNA of the human hinge protein has been cloned and its nucleotide sequence was determined. The deduced primary structure of the amino-terminal presequence consists of 13 amino acid residues, of which 4 amino acids are acidic and only one is basic. Since the presequences of most other precursors are rich in basic amino acids, this sequence is unique for targeting mitochondria. Expression of the gene was repressed in the presence of a phorbol ester in human promyelocyticleukemia cells (HL-60), and this repression was greater than that of the ADP/ATP translocator. These findings suggest that the hinge protein, the expression of which is well regulated, is imported into mitochondria via a specific pathway.

Mitochondria; Presequence; Hinge protein; Gene expression; (HL-60 cell)

1. INTRODUCTION

The hinge protein is a minor subunit of ubiquinol-cytochrome-*c* reductase (bc_1 complex or complex III) in the respiratory chain, and is indispensable for the formation of the cytochrome c_1-*c* complex [1,2]. Like most mitochondrial proteins, the hinge protein is encoded by a nuclear genome, synthesized outside the mitochondria and then imported into the mitochondria [3]. However, its role is not fully understood. Recently, Kim et al. [4] reported that the hinge protein functions as a regulator of the respiratory chain.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number Y00764

It would be interesting to know the mechanism of regulation of expression of this regulatory protein in mammalian mitochondria. For this purpose, it is essential to clone its gene.

Here, we report the molecular cloning of cDNA for the human hinge protein. The deduced amino acid sequence showed that the presequence in the precursor is unusually acidic. The sensitive repression of transcription of the hinge protein in human promyelocytic leukemia cells (HL-60) [5] by a phorbol ester is also reported.

2. MATERIALS AND METHODS

2.1. *Oligonucleotide*

For cloning of the human hinge protein, an oligonucleotide (35-mer), d(GGIGAICCIAAI-GAIGAIGAIGAIGAIGAIGAIGA), which encodes a peptide, GDPKEEEEEEEE, was syn-

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thesized in an automatic DNA synthesizer model 380B (Applied Biosystems, CA, USA) and purified by reverse-phase column chromatography (YMC Pack, AM 313 ODS) in a high-performance liquid chromatography system.

The 5′-end was labelled with [^{32}P]ATP and T_4 nucleotide kinase, and the labelled oligonucleotide was purified on a Nensorb^{TM}20 column (NEN Research Products). The specific activity of the preparation was approx. 1000 Ci/mmol.

2.2. *Cloning*

A human fibroblast cDNA library, constructed in an expression vector, pcD [6], was a kind gift from Dr Okayama (NIH). The library was screened using the synthetic oligonucleotide described above as a hybridization probe. Hybridization was performed at 37°C for 12 h in 2 × SSC, 5 × Denhart's solution, 0.15 mg/ml heat-denatured salmon sperm DNA with the labelled oligonucleotide and then the filter was washed at room temperature for 2 h, at 37°C for 1 h in 2 × SSC and 0.5% SDS and at 45°C for 30 min in 1 × SSC and 0.1% SDS [7]. The positive colonies were purified by successive colony hybridization. The clones were identified by nucleotide sequencing [8,9] and direct comparison of the deduced amino acid sequence with the protein sequence of the bovine hinge protein [10].

The procedure for the cDNA for the human ADP/ATP translocator will be reported elsewhere. The nucleotide sequence was identical with the reported sequence for human ADP/ATP translocator cDNA [11].

2.3. *Nucleotide sequence*

The nucleotide sequence was determined by the dideoxy chain termination method [8,9]. The plasmid was digested with *Xho*I, treated with *Bal*31 nuclease for various times at 30°C, and end-filled with Klenow enzyme. Insert DNAs of various lengths were fractionated by agarose gel electrophoresis, and ligated into M13 mp18 RFI digested with *Sma*I, and dephosphorylated with calf intestinal phosphatase. Nucleotide sequences were determined by the dideoxy-nucleotide chain termination method using single-stranded M13 phage DNA with an insert as a template [8,9]. Computer analysis was performed with a Genetyx program (SDC, Tokyo).

2.4. *Culture of HL-60 with or without TPA*

HL-60 cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum at 37°C under 5% CO_2 in air. Cells were treated with 20 ng/ml TPA (12-*O*-tetradecanoylphorbol-13-acetate) (Sigma) for two days and lysed with 6 M guanidine thionate and promptly used for RNA preparation [12].

2.5. *Northern blotting*

An RNA fraction containing poly(A) was prepared from HL-60 cells by the method as described [12]. Denatured RNA was applied to 1% agarose gel and transferred to a nylon membrane (Hybond N, Amersham). Northern blotting was performed as described by Davis et al. [7]. The probe was prepared by universal labelling of M13 single-stranded DNA harboring the cDNA of the hinge protein and ADP/ATP translocator lacking a poly(A) tail.

3. RESULTS AND DISCUSSION

3.1. *Molecular cloning*

The bovine hinge protein has a unique segment of eight glutamate residues in the amino-terminal region [10] which is homologous with the yeast 17-kDa subunit of complex III [13]. Therefore, we made an oligonucleotide that encodes the amino-terminal region as a cloning probe as described in section 2.

Of 50000 colonies, we picked 15 positive colonies and purified them by successive colony hybridization. The plasmid was purified from the colony that showed the strongest signal in hybridization. The *Pst*I-*Xho*I fragment was isolated, ligated into M13 replicative form DNA and then sequenced for identification.

3.2. *Nucleotide sequence and deduced primary structure*

Fig.1 shows the restriction map of the cloned cDNA. Most of the deduced amino acids were the same as in the bovine sequence [10], as shown in fig.2. In the mature forms, 95% of the amino acid residues were identical. Since a termination codon (TAG) was followed by an initiation codon (ATG) in the open reading frame, we considered that the first 13 amino acids corresponded to the transient presequence as shown in fig.2. The presequence

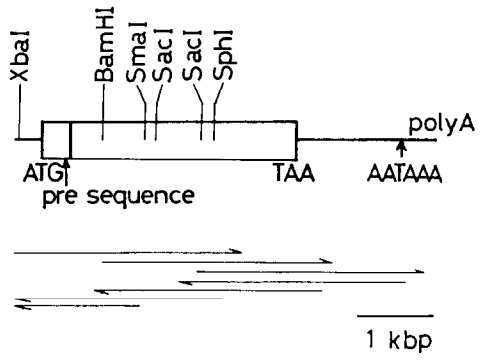

Fig.1. Restriction map and sequence strategy. The nucleotide sequence was determined by the dideoxy chain termination method using fragments subcloned in the phage M13 mp18. The arrows indicate the parts of the sequence determined by these fragments. The coding region is boxed.

was extremely acidic and highly hydrophilic. The amino-terminal region of the yeast 17-kDa protein, which is a counterpart of the hinge protein, is also acidic, but still has 8 basic amino acid residues [13]. In contrast, the human hinge protein has only one basic amino acid residue. Recently, the common properties of the presequences of precursors of mitochondrial proteins have been investigated [15–19]. Extensive investigations have shown that a cluster of basic residues in the conformation plays a role in targeting and transport of proteins [20,21]. However, the presequence of the hinge protein was very different from those of common mitochondrial proteins. The hinge protein is considered to be located on the inner-membrane facing the intermembrane space. Thus, the hinge protein may penetrate the outer mitochondrial membrane but not the inner membrane. Since the

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                                                                    1                  24
GGG.GGG.CTC.GTG.TTG.AAT.CTA.GAA.CCG.TAG.CCA.GAC.ATG.GGA.CTG.GAG.GAC.GAG.CAA.AAG
                                        ***          Met-Gly-Leu-Glu-Asp-Glu-Gln-Lys
                                                     pre-sequence
                                                                                   84
ATG.CTT.ACC.GAA.TCC.GGA.GAT.CCT.GAG.GAG.GAG.GAA.GAG.GAA.GAG.GAG.GAA.TTA.GTG.GAT
Met-Leu-Thr-Glu-Ser-Gly-Asp-Pro-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Leu-Val-Asp
    cleavage site                    Lys(bovine)
                                                                                  144
CCC.CTA.ACA.ACA.GTG.AGA.GAG.CAA.TGC.GAG.CAG.TTG.GAG.AAA.TGT.GTA.AAG.GCC.CGG.GAG
Pro-Leu-Thr-Thr-Val-Arg-Glu-Gln-Cys-Glu-Gln-Leu-Glu-Lys-Cys-Val-Lys-Ala-Arg-Glu

                                                                                  204
CGG.CTA.GAG.CTC.TGT.GAT.GAG.CGT.GAT.TCC.TCT.CGA.TCA.CAT.ACA.GAA.GAG.GAT.TGC.ACG
Arg-Leu-Glu-Leu-Cys-Asp-Glu-Arg-Asp-Ser-Ser-Arg-Ser-His-Thr-Glu-Glu-Asp-Cys-Thr
                                                        Gln(bovine)
                                                                                  264
GAG.GAG.CTC.TTT.GAC.TTC.TTG.CAT.GCG.AGG.GAC.CAT.TGC.GTG.GCC.CAC.AAA.CTC.TTT.AAC
Glu-Glu-Leu-Phe-Asp-Phe-Leu-His-Ala-Arg-Asp-His-Cys-Val-Ala-His-Lys-Leu-Phe-Asn
            Leu(bovine)
                                                                                  324
AAC.TTG.AAA.TAA.ATG.TGT.GGA.CTT.AAG.TTG.CAC.CCC.AGT.CTT.CAT.CAT.CTG.GGC.ATC.AGA
Asn-Leu-Lys-***
Ser(bovine)
                                                                                  384
ATA.TTT.CCT.TAT.GGT.TTT.GGA.TGT.ACC.ATT.TGT.TTC.TTA.TTT.GTG.TAA.CTG.TAA.GTT.CAC
                                                                                  444
ATC.AAC.CTC.ATG.GGT.TTG.GCT.TGA.GGC.TGG.TAG.CTT.CTA.TGT.AAT.TCG.CAA.TGA.TTC.CAT

CTA.AAT.AAA.AGT.TCT.ATG.ATC.TGC.AAA.AAA.AAA.
   poly A signal
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Fig.2. Nucleotide sequence and deduced amino acid sequence. The amino acids of the bovine sequence differing from those of the human one are noted under the primary structure of the human sequence. The poly(A) additional signal, AATAAA, and the putative presequence peptide are underlined. The acidic amino acids in the presequence are boxed.

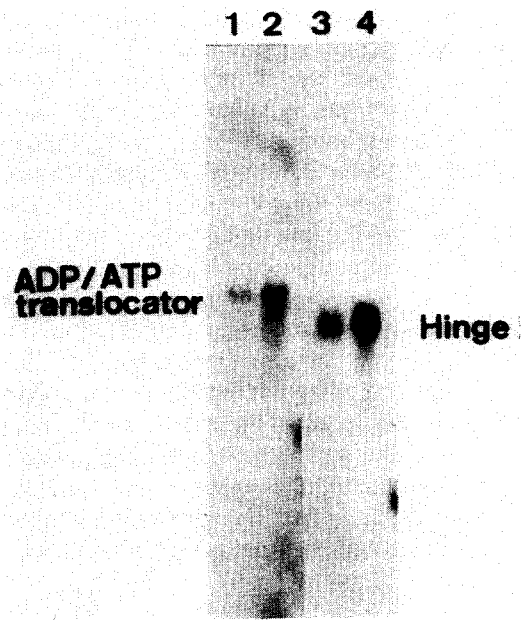

Fig.3. Northern blotting of mRNA from HL-60 with or without treatment with TPA. HL-60 cells (10^6 cells) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence or absence of 20 nM TPA for 48 h. The RNAs were isolated and the RNA fraction containing poly(A) (5 μg) was fractionated on 1% agarose gel. Blotting and hybridization were performed as described in section 2. Hybridization was performed with the ^{32}P-labelled ADP/ATP translocator cDNA fragment. After the first experiment, the filter was washed at 60°C for 1 h to remove the first probe, and then hybridized with the hinge protein gene. Lanes: 1, ADP/ATP translocator, with TPA; 2, ADP/ATP translocator, without TPA; 3, hinge protein, with TPA; 4, hinge protein without TPA.

presequences show common features, including the outer-membrane [17] and inter-membrane space proteins [18], the presequence of the hinge protein is quite unique. This finding suggests that the hinge protein is imported into mitochondria via a specific 'receptor'.

3.3. *Expression of the gene*

As shown in fig.3, Northern blotting indicated a single species of messenger RNA for the hinge protein. Recently, Battini et al. [11] reported that gene expression of the ADP/ATP translocator was repressed in HL60 treated with TPA. Fig.3 shows the reduction of the mRNA by TPA for ADP/ATP translocator in HL-60. In addition, the mRNA of the hinge protein was reduced more dramatically by TPA treatment. Further investigations are necessary to determine whether repression of the gene or stability of the mRNA of the hinge protein is the more sensitive to TPA treatment. The content of the mRNA for the hinge protein was affected the most. These findings suggest that gene expression of the hinge protein, a regulator, is well regulated and that the protein is transported via a specific 'receptor' using the extremely acidic presequence.

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