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The cleavable prepiece of an imported mitochondrial protein is sufficient to direct cytosolic dihydrofolate reductase into the mitochondrial matrix

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The cleavable prepiece of the precursor to yeast cytochrome *c* oxidase subunit IV (an imported mitochondrial protein) was attached to the amino-terminus of mouse dihydrofolate reductase (a cytosolic protein) by gene fusion. The resulting fusion protein was imported into the matrix of isolated, energized yeast mitochondria and cleaved to a polypeptide whose size was similar to that of authentic dihydrofolate reductase.

Mitochondrial protein import *Yeast mitochondria* *Gene fusion* *Dihydrofolate reductase* *Precursor protein*

1. INTRODUCTION

Most imported mitochondrial polypeptides are initially synthesized as larger precursors with amino-terminal 'prepieces' which are proteolytically removed in the mitochondrial matrix [1,2]. When these prepieces are removed by adding processing protease isolated from the matrix, the resulting mature polypeptides are no longer imported into isolated mitochondria [3]. The cleavable prepieces are, thus, necessary for import to occur. Do these prepieces carry all the information for directing the precursors into mitochondria or is some information also provided by that part of the precursor chain which is retained in the mature polypeptide?

The experiments described here show that the cleavable prepiece of the yeast cytochrome *c* oxidase subunit IV precursor is sufficient to direct a mouse cytosolic enzyme into the yeast mitochondrial matrix.

2. MATERIALS AND METHODS

2.1. *Gene fusion*

The cleavable prepiece of the yeast cytochrome *c* oxidase subunit IV precursor [4] was attached to the amino-terminus of mouse dihydrofolate reductase [5] as outlined in fig.1. The starting material was plasmid pDS5/2; this plasmid was constructed by authors in [6] and permits the in vitro transcription-translation of cloned genes. By removing one of the two *Eco*RI sites and inserting the cloned gene for cytochrome *c* oxidase subunit IV, plasmid pDS5/2-1-CoxIV was obtained [7]. The single *Xho*I site of plasmid pDS5/2-1-CoxIV was first removed by cutting with *Xho*I, filling in the ends with the large ('Klenow') fragment of DNA polymerase, and religation. The reclosed plasmid was then cut with *Eco*RV at a site corresponding to amino acid residue 23 of the subunit IV prepiece (total length of the prepiece: 25 amino acids). An *Xho*I linker (New England Biolabs) was attached, the plasmid was recircularized by ligation, and recut with *Xho*I to remove excess linker. The ends were filled in as above, the linear piece of DNA was cut with *Hin*dIII, and the larger of the two resulting fragments was isolated [8]. This fragment contained the coding sequence for most of the subunit IV prepiece. To obtain the dihydrofolate reductase gene, pDS5/2-1 was cut with *Bam*HI, the ends were filled in as above, the linearized plasmid was cut with *Hin*dIII, and the smaller of the two fragments was isolated [8]. It

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was ligated to the large fragment obtained from pDS5/2-1-CoxIV (cf. above); the resulting plasmid pDS5/2-1-PCoxIV-DHFR contained the desired fused gene.

2.2. *Miscellaneous*

Recombinant DNA methods [9], assaying import by isolated yeast mitochondria [3], SDS-polyacrylamide gel electrophoresis and fluorography [3], in vitro transcription-translation of genes cloned into pDS5/2-1 [6,7], isolation of processing protease from the yeast mitochondrial matrix [10], subfractionation of yeast mitochondria [11] and quantitative immune blotting [12] have been described. L-[^{35}S]Methionine (>1000 Ci/mmol) was from Amersham, England.

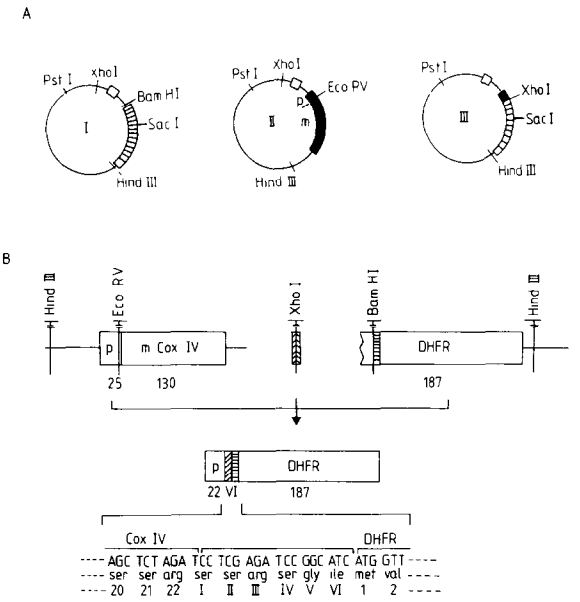

Fig.1. Attaching the cleavable prepiece of the yeast cytochrome *c* oxidase subunit IV precursor to the amino-terminus of mouse dihydrofolate reductase. See text for details. (A) Restriction maps of plasmids. I, pDS5/2-1 (starting plasmid); II, pDS5/2-1-CoxIV (plasmid containing the subunit IV gene); III, pDS5/2-1-PCOXIV-DHFR (plasmid containing the fused gene used here). Open, filled and shaded bars denote DNA sequences encoding the bacteriophage T_5 promotor, yeast subunit IV precursor, and mouse dihydrofolate reductase, respectively. p and m in II indicate DNA sequences encoding the prepiece and the mature part of subunit IV (Cox IV). DHFR, dihydrofolate reductase. (B) Gene fusion protocol. Arabic numerals underneath the bars denote amino acids counted from the amino-termini of either the prepiece (p) or the 'mature' part (m) of cytochrome *c* oxidase subunit IV (CoxIV) or the amino-terminus of mouse dihydrofolate reductase (DHFR, [5]). Roman numerals identify amino acids introduced by gene manipulation. The horizontally shaded part in the DHFR gene denotes 11 extra nucleotides derived from the vector.

3. RESULTS

3.1. *The fusion protein*

The protein encoded by the fused gene (fig.1) contains 3 regions. Starting at the amino-terminus, these are as follows: (i) 22 (out of the 25) amino acids from the cleavable prepiece of the subunit IV precursor; (ii) 6 amino acids introduced by gene fusion; (iii) the entire polypeptide chain of mouse dihydrofolate reductase (187 amino acids). Dihydrofolate reductase is located in the cytoplasm of rodent cells [13]. Since the fusion protein lacks the last 3 amino acids of the subunit IV prepiece, it no longer contains the cleavage site leu(−1)–gln(+1) of the authentic subunit IV precursor. The structure of the fused gene was verified by restriction endonuclease digestion (fig.2). While this method is generally not applicable to products derived by ligation of blunt-ended fragments, the blunt-end ligation outlined in fig.1 generates a new *Xho*I site. This site was indeed found (fig.2, lane 3). When the fused gene was transcribed with *E. coli* RNA polymerase and the resulting mRNA capped and translated in a nuclease-treated reticulocyte lysate [6,7], the radiolabeled polypeptide product had an apparent M_r of 25000 (fig.3). This agrees well with the predicted value of 24611 (22 amino acids of the subunit IV prepiece = 2424; 6 extra amino acids = 587; mouse dihydrofolate reductase = 21600).

3.2. *The fusion protein is cleaved by mitochondrial processing protease*

The chelator-sensitive processing protease isolated from the yeast mitochondrial matrix only cleaves native mitochondrial precursor proteins; it is inactive towards denatured precursors, mature mitochondrial proteins, or non-mitochondrial proteins [10]. Since the enzyme cleaves a variety of peptide bonds [4], it appears to recognize some three-dimensional feature(s) of the native precur-

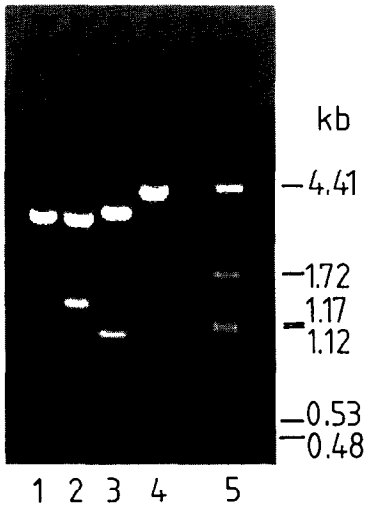

Fig.2. Restriction digests of plasmid pDS5/2-1-PCoxIV-DHFR (containing the fused gene used in this study). The experimentally determined size of each DNA fragment agreed within experimental error with the size predicted from the DNA sequence. 1, no restriction endonuclease; 2, *pstI/SacI*; 3, *PstI/XhoI*; 4, *PstI/Eco*RV; 5, molecular size markers (*Hin*dIII digest of pBR 322-SV40).

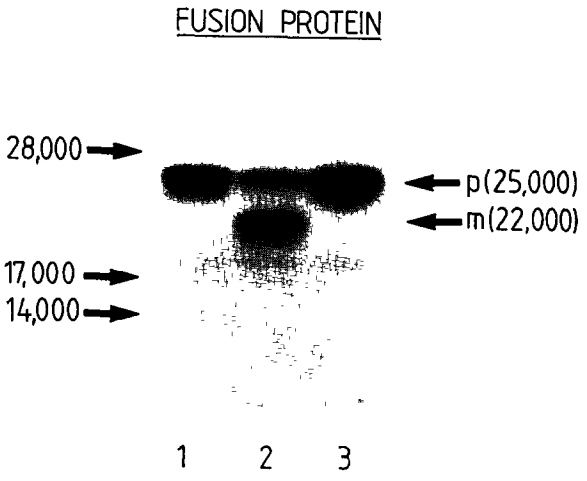

Fig.3. The fusion protein is cleaved by purified processing protease from the yeast mitochondrial matrix. The fusion protein was synthesized in vitro in the presence of [^{35}S]methionine and incubated for 30 min at 30°C with or without matrix protease (6 μg, purified through step 2 [10]). After incubation, the mixture was analyzed by dissociation in SDS at 95°C, SDS-12% polyacrylamide slab gel electrophoresis, and fluorography [9]. 1, no protease; 2, with protease; 3, with protease and 2 mM 1,10-phenanthroline. p and m, precursor- and processed forms of the fusion protein; the arrows and numbers on the left indicate the positions and sizes (in d) of standard proteins and the numbers in parentheses on the right the experimentally determined sizes (in d) of the fusion protein and its processed form.

sor. This protease cleaves the fusion protein (fig.3) cleavage is blocked by 1,10-phenanthroline, a fairly specific inhibitor of the enzyme. The size of the processed polypeptide (M_r 22000) is closely similar to that of authentic radiolabeled dihydrofolate reductase (M_r 21600). Processing decreases the size of the fusion protein by about 3 kDa. This is consistent with a removal of the subunit IV prepiece (including, perhaps, some of the 6 extra amino acids that had been attached to the amino-terminus of dihydrofolate reductase). The exact cleavage site is currently being determined by amino acid sequencing.

3.3. *The fusion protein is imported into the matrix, and processed, by isolated mitochondria*

When the radiolabeled fusion protein was incubated with energized yeast mitochondria, it was processed and the processed form was resistant to externally added proteinase K. Resistance was abolished by disrupting the mitochondrial membranes with Triton X-100. Deenergized mitochondria bound the fusion protein, but neither imported nor processed it (fig.4). Time-course experiments (not shown) indicated that the fusion protein was imported by isolated mitochondria at a rate which was identical (within a factor of two) to that found with the authentic subunit IV precursor.

The imported processed protein is located in the matrix: it is inaccessible to added proteinase K not only in mitochondria, but also in mitochondria whose outer membrane had been disrupted ('mitoplasts'; fig.5). Immune blotting confirmed that the mitoplasts had lost virtually all of their intermembrane space marker, cytochrome b_2; removal of the outer membrane barrier had thus been essentially complete. In additional subfractionation experiments (not shown) the processed fusion protein cofractionated with the matrix marker, citrate synthase.

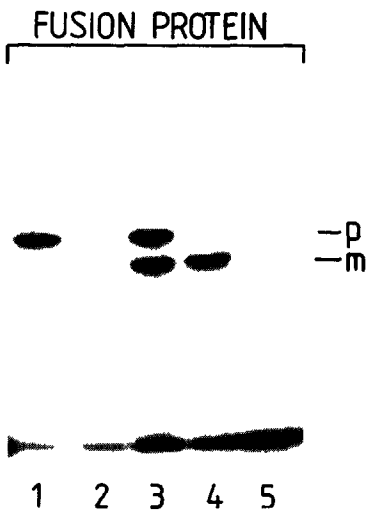

Fig.4. The fusion protein is imported, and processed, by isolated energized mitochondria. Reticulocyte lysate (15 μl, containing the ^{35}S-labeled fusion protein) was incubated for 30 min at 30°C in a final volume of 200 μl with 140 μg yeast mitochondria, 40 mM KCl and the ATP-regenerating system, etc. described in [3]. To deenergize the mitochondria, 1 μg valinomycin/ml was added. Where indicated, the mixture was incubated for additional 30 min at 0°C with 250 μg proteinase K/ml, followed by addition of phenylmethylsulfonyl fluoride to 1 mM. Pelleted mitochondria [3] and supernatant were analyzed as in fig.3. 1, deenergized mitochondria; 2, deenergized mitochondria, proteinase K; 3, energized mitochondria; 4, energized mitochondria, proteinase K; 5, energized mitochondria, proteinase K and 1% Triton X-100. p and m, precursor- and processed forms of the fusions protein.

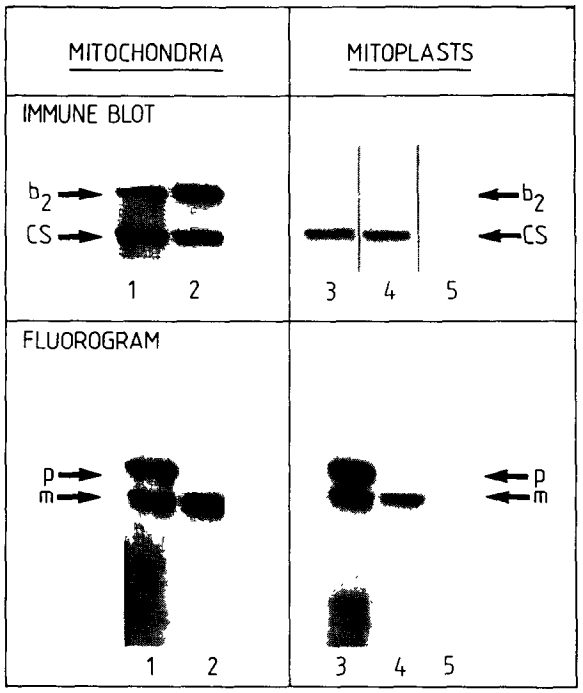

Fig.5. The fusion protein is imported into the matrix space. Energized yeast mitochondria were allowed to import the radiolabeled fusion protein (cf. fig.4) and divided into 5 aliquots which were: (a) directly dissociated in SDS; (b) treated with proteinase K and then dissociated; (c) converted to mitoplasts and dissociated; (d) converted to mitoplasts, treated with proteinase K, and dissociated; (e) converted to mitoplasts, treated with proteinase K in the presence of 1% Triton X-100, and dissociated. Each dissociated sample was subjected to SDS-12% polyacrylamide gel electrophoresis and analyzed by fluorography for radiolabeled fusion protein or by immune blotting for the intramitochondrial marker proteins cytochrome b_2 (intermembrane space) and citrate synthase (matrix). 1, mitochondria; 2, mitochondria, proteinase K; 3, mitoplasts; 4, mitoplasts, proteinase K; 5, mitoplasts, proteinase K, Triton X-100.

4. DISCUSSION

This study shows for the first time that a cytosolic protein can be transported across membranes if it fused to a cleavable prepiece derived from an imported mitochondrial protein. This transport is remarkable for 3 reasons. First, it can occur post-translationally, i.e., with the folded fusion protein. Indeed, the unusual trypsin-resistance of the mouse dihydrofolate polypeptide is retained by the corresponding segment of the fusion protein (not shown). Second, the fusion protein is transported into mitochondria nearly as rapidly as the mitochondrial precursor from which the cleavable prepiece had been derived. Third, the cytosolic polypeptide did not become stuck across one of the mitochondrial membranes, but was transported all the way across these two membranes into the matrix space.

These results permit several important conclusions. Firstly, they show that the cleavable prepiece of the subunit IV precursor contains all the necessary information for importing an attached cytosolic protein into mitochondria. In fact, only 22 amino acids (out of the 25 present in the prepiece) are sufficient. This strongly suggests that

the polypeptide attached to the prepiece does not actively contribute to the transport process. Further work will determine whether this conclusion can be extended to other prepieces and to other cytosolic proteins. Some caution seems indicated, since our data are at variance with those of authors in [14]; these authors fused amino-terminal regions derived from the precursor to the yeast F_1-ATPase β-subunit to *E. coli* β-galactosidase and found that the fusion proteins were targeted to mitochondria only if they contained at least 139 amino-terminal residues derived from the β-subunit precursor. Since the cleavable prepiece of that precursor contains only about 20 residues [15], part of the 'mature' β-subunit appears to participate in the targeting process. Alternatively, part of the mature β-subunit may be required to keep the prepiece suitably exposed on the fusion protein.

Secondly, our data suggest that the cleavable prepiece of the subunit IV precursor can fold independently of the polypeptide chain to which it is attached. Since the matrix protease does not cleave denatured precursors [10] or dihydrofolate reductase ([7]; cf. also fig.3), cleavage of the fusion protein must involve interaction of the protease with the properly folded subunit IV prepiece. Once this interaction has occurred, the actual cleavage is sequence-unspecific since the fusion protein lacks the cleavage site of the authentic subunit IV precursor.

Thirdly, it now appears rewarding to study the conformation and the biological activity of cleavable prepieces which have been chemically synthesized in large amounts. Such studies would open the field of mitochondrial protein import to physico-chemical methods and might shed light on how prepieces perform their function.

Finally, it may be possible to import a great variety of non-mitochondrial proteins into mitochondria by attaching to these proteins prepieces which are again removed inside the mitochondria.

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REFERENCES

[1] Schatz, G. and Butow, R.A. (1983) Cell 32, 316–318.
[2] Hay, R., Böhni, P. and Gasser, S.M. (1984) Biochim. Biophys. Acta 779, 65–87.
[3] Gasser, S.M., Daum, G. and Schatz, G. (1982) J. Biol. Chem. 257, 13034–13041.
[4] Maarse, A.C., Van Loon, A.P.G.M., Riezman, H., Gregor, I., Schatz, G. and Grivell, L.A. (1984) EMBO J. 3, in press.
[5] Stone, D. and Phillips, A.W. (1977) FEBS Lett. 74, 85–87.
[6] Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. and Bujard, H. (1984) EMBO J. 3, in press.
[7] Hurt, E.C., Pesold-Hurt, B. and Schatz, G. (1984) EMBO J. 3, in press.
[8] Dretzen, G., Bellard, P., Sassone-Corsi, P. and Chambon, P. (1981) Anal. Biochem. 112, 295–298.
[9] Riezman, H., Hase, T., Van Loon, A.P.G.M., Grivell, L.A., Suda, K. and Schatz, G. (1983) EMBO J. 2, 2161–2168.
[10] Böhni, P., Daum, G. and Schatz, G. (1983) J. Biol. Chem. 258, 4937–4943.
[11] Daum, G., Böhni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
[12] Haid, A. and Suissa, M. (1983) Methods Enzymol. 96, 192–205.
[13] Wang, F.K., Koch, J. and Stockstad, F.L.R. (1967) Biochem. Z. 346, 458–467.
[14] Douglas, M.G., Geller, B.L. and Emr, S.D. (1984) Proc. Natl. Acad. Sci. USA 81, 3983–3987. Also: Emr, S.D. and Douglas, M.G., personal communication.
[15] Maccechini, M.-L., Rudin, Y., Blobel, G. and Schatz, G. (1979) Proc. Natl. Acad. Sci. USA 76, 343–347.