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SYNTHESIS AND INTRACELLULAR TRANSPORT OF CYTOCHROME OXIDASE SUBUNIT IV AND ADP/ATP TRANSLOCATOR PROTEIN IN INTACT HEPATOMA CELLS

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SUMMARY: The biosynthesis of two mitochondrial membrane proteins - subunit IV of cytochrome oxidase and ADP/ATP translocator protein was studied in intact ascites hepatoma cells. Using pulse-chase labeling and rapid cell fractionation it was possible to identify the precursoric forms of these inner mitochondrial membrane proteins. It was found that the subunit IV of cytochrome oxidase is synthesized in the cytoplasm of mammalian cells in the form of a larger precursor while ADP/ATP translocator protein is synthesized in the form that is electrophoretically undistinguishable from the mature membrane integrated form.

The major part of mitochondrial proteins is synthesized on the cytoplasmic ribosomes and consequently transported to the mitochondria /1/. With few exceptions these imported proteins are initially synthesized in the form of larger precursors /2,3/. The characterization of these precursors and the events involved in their posttranslational fate have been extensively studied in yeast and fungi /2-7/. Most of the corresponding studies with mammalian cells were performed using heterologous cell-free protein synthesizing systems /8-11/. Physiologically more relevant systems have been employed only in a limited extent. Consequently, the information on the intracellular synthesis, transport and processing of mitochondrial proteins in intact mammalian cells are restrained to two soluble mitochondrial

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enzymes - ornithine transcarbamylase and carbamoyl-phosphate synthetase /12-15/. One of the main technical obstacles in experiments with whole mammalian cells resides in relatively low rates of protein synthesis resulting in a low labeling of the proteins examined. In the present study we took advantage of the high rate of protein synthesis in rapidly growing rat hepatoma for the study of biosynthesis of two mitochondrial membrane proteins - subunit IV of cytochrome oxidase and ADP/ATP translocator protein in whole mammalian cells. The precursoric forms of these proteins in whole cells were identified, and their behavior in pulse-chase labeling experiments was established.

METHODS

The maintenance, propagation and harvesting of Zajdela ascites cells /16/ as well as the conditions for incubation of isolated cells /17/ were as described. In pulse-chase experiments the density of the cells in the incubation medium was increased to 9×10^7 cells per ml (about 30 mg protein/ml) and the chase was initiated by addition of an equal volume of 37 °C warm incubation medium supplemented with 15 mM unlabeled methionine.

For immunoadsorption of mitochondrial membrane proteins the cytosolic and particulate fraction from the [^{35}S]methionine labeled cells were prepared according to /18/ with some modifications /15/. 0.1 ml of cell suspension (about 10^7 cells) were mixed with 0.4 ml of solution containing 0.25 M sucrose, 3 mM EDTA, 20 mM HEPES, 5 mM o-phenantroline, 200 units/ml Trasylol (Bayer) and 1 mg/ ml digitonine (final pH 7.4). After 2 min at 0 °C, the mixture was centrifuged 1 min at 12 000g. The supernatant and the pellet were rapidly removed and precipitated in cold by 15% trichloroacetic acid. After centrifugation the precipitates were washed with cold water, solubilized with 2% SDS and diluted 10 times with 2% Triton X-100, phosphate--buffered saline, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (pH 7.4). The lysates were clarified by centrifugation and used for immunoadsorption with antisera against rat mitochondrial proteins and Sepharose-protein A as described /17/. In some experiments whole labeled cells were precipitated by 15% trichloroacetic acid and then processed as above. Holo-cytochrome oxidase was immunoadsorbed from Triton X-100 solubilized labeled cells as described previously /17/.

Raising of antisera in rabbits /17,19/, solubilization of the immunoadsorbed material /20/, electrophoresis in SDS polyacrylamide /21/ and fluorography of gel slabs /22/ were performed according to published procedures. [^{35}S]methionine />1000 Ci/mmol/ was from Amersham /England/.

RESULTS

Zajdela hepatoma cells incubated under the conditions found to be optimal for protein synthesis in isolated hepatocytes /17/

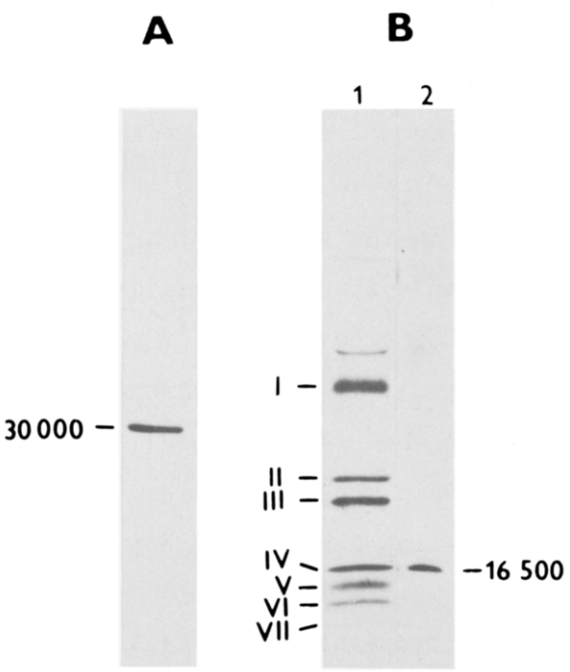

Fig.1. [^{35}S]methionine labeled peptides of rat hepatoma cells immunoadsorbed with antisera against rat mitochondrial proteins. /A/ -ADP/ATP translocator protein; /B/ -holo-cytochrome oxidase (1), and subunit IV of the enzyme (2). Cells were labeled for 60 min with [^{35}S]methionine (0.1 mCi/ml) and then 10 min chased with 7.5 mM unlabeled methionine. The labeled cells were processed as described in Methods and subjected to immunoadsorption with corresponding antisera. The immunoadsorbed radioactive proteins were electrophoresed and vizualized by fluorography.

incorporated 5 to 10 times more [^{35}S]methionine than did the isolated hepatocytes. Newly synthesized [^{35}S]methionine labeled mitochondrial membrane proteins in whole hepatoma cells were identified using antisera raised against the proteins studied. ADP/ATP translocator protein, cytochrome oxidase and its subunit IV immunoadsorbed from hepatoma cells labeled continuously for 1 hour with [^{35}S]methionine are shown in Fig.1. All subunits of cytochrome oxidase as well as ADP/ATP translocator protein became intensively labeled during this incubation period. Fig.1 also shows that the antisera used were of satisfactory specificity.

In order to detect the form in which subunit IV of cytochrome oxidase and ADP/ATP translocator protein are synthesized on the

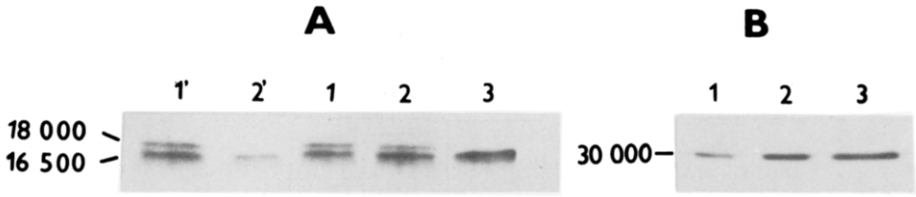

Fig.2. Cytochrome oxidase subunit IV and ADP/ATP translocator protein immunoadsorbed from the cells labeled for different time. Cells were pulsed with [^{35}S]methionine (0.5 mCi/ml) for 7.5 min /lanes 1 and 1'/ and 15 min /lane 2/ or were pulsed for 15 min and then chased for 15 min in the presence of an excess of unlabeled methionine /lane 3/. In lane 2' the cells were labeled as described under Fig.1. At the times indicated aliquots of labeled cells were precipitated with 15% trichloroacetic acid and treated as described in Methods before addition of anti--subunit IV /A/ or anti-ADP/ATP translocator sera /B/. Only the relevant part of the gel fluorogram is shown.

cytoplasmic ribosomes in intact hepatoma cells [^{35}S]methionine labeled peptides immunoadsorbed from pulse labeled cells and those immunoadsorbed from continuously or pulse-chase labeled cells were compared. Besides the mature subunit IV (Mr 16 500), a labeled peptide with molecular weight 18 000 was immunoadsorbed with anti-subunit IV serum from pulse labeled cells /Fig.2 A/. The radioactivity incorporated into subunit IV evidently increased during the pulse while that incorporated into the larger peptide remained virtually unchanged. Following 15 min chase with unlabeled methionine the radioactivity in the mature subunit IV showed further increase. At the same time the label in the larger molecular weight component disappeared completely. Using anti--ADP/ATP translocator serum a single labeled peptide was immunoadsorbed from both pulse and pulse-chase labeled cells with molecular weight corresponding to that of the isolated enzyme /Fig.2 B/.

In order to determine the intracellular distribution of the newly synthesized mitochondrial membrane proteins [^{35}S]methionine pulse or pulse-chase labeled hepatoma cells were fractionated to cytosol and particulate fraction. Both fractions were further subjected to immunoadsorption with the antisera and the immunoadsorbed radioactivity was analysed by SDS polyacrylamide gel

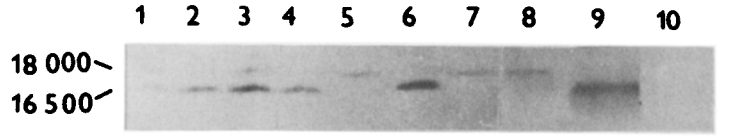

Fig.3. Subunit IV immunoadsorbed from the cellular fractions of pulse and pulse-chase labeled hepatoma cells. Hepatoma cells were labeled as described under Fig.2. At the times indicated aliquots of labeled cells were removed and fractionated as described in Methods. Both the cytosolic and the particulate fraction subjected to immunoadsorption followed by electrophoresis and fluorography. Lanes 1,2,3, - 5, 10 and 15 min pulse labeled particulate fractions; lanes 5,7,8, - labeled peptides immunoadsorbed from the 5, 10 and 15 min pulse labeled cytosol; lanes 4,6,9, - labeled peptides from particulate fraction of cells labeled for 5, 10 and 15 min and chased for 10 min. No radioactivity was immunoadsorbed from the pulse-chase labeled cytosol fractions /lane 10/.

electrophoresis. It was found that the cytosolic fraction from pulse labeled cells contained the larger labeled peptide (Mr 18 000) immunoadsorbed with anti-subunit IV serum but it did not contain labeled mature subunit IV /Fig.3, lanes 5,7,8/. The corresponding particulate fractions from pulse labeled cells contained the both labeled peptides /Fig.3, lanes 1-3/. Similarly, as in whole cells the radioactivity of the larger peptide obtained from the cytosol did not change significantly during the pulse period. After short chase with unlabeled methionine the only labeled peptide immunoadsorbed with anti-subunit IV serum from particulate fraction was mature subunit IV /Fig.3, lanes 4,6,9/. No radioactivity was immunoadsorbed from the corresponding cytosol fractions.

Anti-translocator serum immunoadsorbed from both pulse labeled cytosol and pulse labeled particulate fraction a single labeled peptide with molecular weight identical to that of the mature membrane integrated protein. However, the labeled peptide from the cytosol and particulate fraction exhibited different behavior upon the chase. The radioactivity in the peptide immunoadsorbed from the cytosol was rapidly cleared out while that from the particulate fraction evidently increased /Fig.4/.

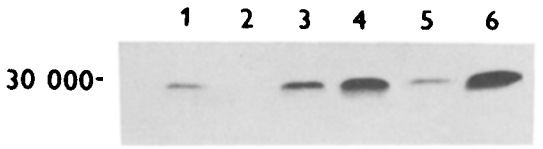

Fig.4. ADP/ATP translocator protein immunoadsorbed from the cellular fractions of pulse and pulse-chase labeled hepatoma cells. Cells were labeled and fractionated as in Fig.3 and the fractions were immunoadsorbed with anti-ADP/ATP translocator serum. Fluorographs of immunoadsorbed material are shown for: Cytosol labeled for 10,15 min /lanes 1,5/; cytosol labeled for 10 min and chased for 10 min /lane 2/; particulate fraction labeled for 10 min /lane 3/ and particulate fraction labeled for 10, 15 min followed by 10 min chase /lanes 4,6/.

To exclude the possibility that the cytosolic fraction of hepatoma cells obtained after digitonine treatment might be contaminated with mitochondrial membrane fragments control experiments were carried out with antisera against intramitochondrially synthesized cytochrome oxidase subunits II and III. The corresponding labeled peptides were found to be present exclusively in the particulate fraction of labeled cells /not shown/.

DISCUSSION

The experiments described in this work represent an attempt to identify the precursoric forms of two inner mitochondrial membrane proteins in whole mammalian cells. The following are the evidences that the labeled peptides shown in Fig.3 -lanes 5,7,8 and Fig.4 -lanes 1,5 represent the cytoplasmically synthesized precursors of cytochrome oxidase subunit IV and ADP/ATP translocator protein: Both labeled peptides i/ were located in the cytosolic fraction of cells pulsed with [^{35}S]methionine, ii/ exhibited a very short life-time in the cytosol, and iii/ interacted with antisera against the respective membrane proteins. Moreover, the radioactivity of the mature integrated forms of the peptides increased during the chase on the expense of the label of the corresponding non-integrated peptides. Our results further show that subunit IV of cytochrome oxidase is synthesized in hepatoma cells in the form of a 1 500 molecular

weight larger peptide resembling the size found for the same protein synthesized in a cell-free system directed with liver poly/A/$^{+}$RNA /8/. These data are inconsistent with a proposal /23/ that extramitochondrially formed subunits of mammalian cytochrome oxidase are synthesized as a polyprotein precursor.

It appears that in lower eucaryotic cells the ADP/ATP translocator protein is an exception of the rule that integral proteins of inner mitochondrial membrane are synthesized as larger precursors /5/. The results of this work show that this holds also for mammalian cells.

The data presented show that the precursoric forms of mitochondrial membrane proteins can be identified in intact mammalian cells using pulse-chase labeling complemented with a rapid cell fractionation method. Along with our previous results on the characterization of mitochondrial translation products /20/ they also demonstrated the suitability of rapidly growing tumors for study of mitochondrial biogenesis in whole mammalian cells.

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