Coupling between proteolytic processing and translocation of the precursor of the F_1-ATPase β-subunit during its import into mitochondria of intact cells

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The intracellular transport of newly synthesized β-subunits of the F_1-ATPase (βF_1) and of newly synthesized ADP/ATP carrier was followed in isolated rat hepatoma cells. As tested by rapid fractionation of [^{35}S]methionine pulse- and pulse-chase-labeled cells and by sensitivity of labeled polypeptides to externally added protease, the import of βF_1 into mitochondria was strongly inhibited by the additional low concentrations of rhodamine 6G (R6G). In contrast, the import of the ADP/ATP carrier into mitochondria was not affected by the inhibitor. The results imply that the proteolytic processing of the precursor of βF_1 is coupled to its translocation across the mitochondrial membrane.

Precursor protein *Mitochondrial protein transport* *Processing protease* *Rhodamine 6G* *F_1-ATPase*

1. INTRODUCTION

The majority of mitochondrial proteins is synthesized on cytoplasmic ribosomes in the form of higher-M_r precursors (review [1]). The final step of the import of the precursors into mitochondria is the proteolytic cleavage of their NH_2-terminal peptide extension. This is performed by a chelator-sensitive proteolytic enzyme located in the mitochondrial matrix [2–4]. The involvement of the protease in the translocation process has been studied in detail using a cell-free system [5]. It was concluded that the cleavage of the NH_2-terminal extension is not necessary for the translocation of precursor proteins across the mitochondrial membrane [5].

We have found that in intact hepatoma cells R6G inhibits the import into mitochondria of several mitochondrial membrane protein precursors [6]. Under these conditions R6G did not exhibit any uncoupling effect. This compound has also been shown to inhibit the proteolytic processing of precursors by a mitochondrial matrix fraction (Kužela, Š., Joste, V. and Nelson, B.D., submitted). In this study we show that the sensitivity to R6G of the import into mitochondria of newly synthesized mitochondrial proteins depends on the presence of a cleavable signal sequence on the precursor. In R6G-inhibited cells the uncleaved precursors accumulated outside the mitochondria prevailing in the cytosol fraction. This implies that, at least in intact cells, the translocation of the precursor of βF_1 across the mitochondrial membrane is coupled to its proteolytic processing.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; βF_1, β-subunit of mitochondrial F_1-ATPase; R6G, rhodamine 6G; pre-βF_1, precursor of β-subunit of mitochondrial F_1-ATPase

2. MATERIALS AND METHODS

The methods for maintenance and isolation of Zajdela hepatoma cells [7] as well as the labeling of the cells with [^{35}S]methionine (spec. act. > 850 Ci/mmol, Amersham) have been described [8]. The preparation of soluble and particulate fractions from digitonin-permeabilized cells was

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performed according to [9] with some modifications [10]. When the sensitivity of newly synthesized mitochondrial proteins to an externally added protease was estimated, the soluble cellular fraction was incubated with 0.1 mg/ml proteinase K (Merck) for 30 min at 4°C. The corresponding particulate fractions were resuspended in 0.25 M sucrose, 5 mM Hepes, 3 mM EDTA, 1 mM *o*-phenanthroline (pH 7.4) to a volume equivalent to that of the soluble fraction and treated with proteinase K as above. The proteolytic digestion was stopped by addition of 2 mM PMSF followed by 15% trichloroacetic acid. The precipitated proteins were solubilized in 5% SDS by sonication, neutralized, heated for 2 min at 100°C, diluted with ice-cold 1% Triton X-100, phosphate-buffered saline, 5 mM EDTA, 1 mM PMSF (pH 7.4), clarified by centrifugation and immunoadsorbed with the appropriate antiserum and Sepharose-protein A (Pharmacia) essentially as in [6,10]. Published procedures for raising the antisera [11], solubilization of the immunoadsorbed labeled antigens [8], electrophoresis in SDS-polyacrylamide [12] and fluorography of the gels [13] were used.

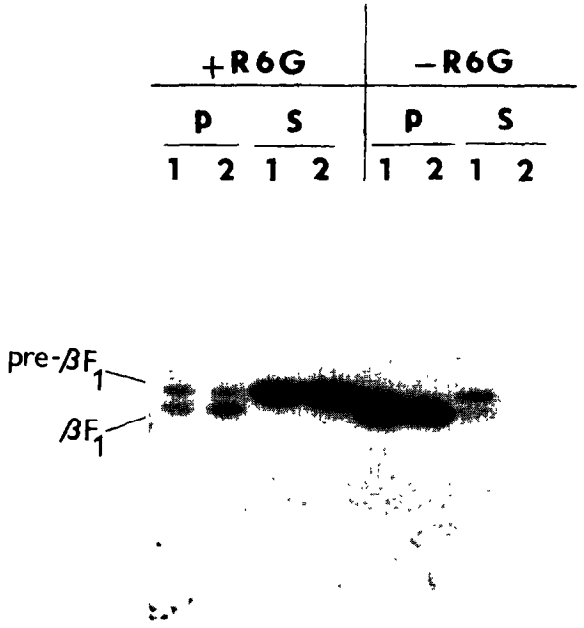

Fig.1. Precursor of βF_1 accumulates in the cytosolic fraction of R6G treated cells. Isolated Zajdela hepatoma cells (30 mg protein/ml) were incubated for 10 min with [^{35}S]methionine (0.25 mCi/ml) and then chased for 10 min with 2 mM unlabeled methionine. Where indicated, 0.65 μg R6G/mg protein was added 5 min before the labeled methionine. At the end of both pulse and chase periods, aliquots of the cell suspension were removed, the cells were rapidly fractionated and the fractions were processed and immunoadsorbed for βF_1 as specified in section 2. (S) Soluble fraction, (P) particulate fraction, (lanes 1) 10 min pulse, (lanes 2) 10 min pulse followed by a 10 min chase.

3. RESULTS AND DISCUSSION

Incubation of isolated Zajdela hepatoma cells with [^{35}S]methionine in the presence of R6G leads to an extensive accumulation of the precursor of βF_1 in the cell cytosol (fig.1). No substantial decrease in the amount of the radioactive pre-βF_1 in the cytosol fraction of R6G-treated cells was observed during a relatively long chase (up to 30 min) with unlabeled methionine indicating a remarkable metabolic stability of the accumulated precursor. A certain amount of the precursor was also present in the particulate membrane fraction of both control and R6G-inhibited pulse-labeled cells. After the chase, precursor was found in the particulate fraction of R6G-treated cells only (fig.1). This sedimentable portion of the precursor might consist either of molecules retained unspecifically with the membranes and/or of molecules associated specifically with the mitochondrial surface. The possibility also exists that this small fraction of precursor is taken up by mitochondria but is not processed in the presence of R6G because of inhibition of the processing protease. This would resemble the findings [5] where unprocessed precursor was found in mitochondria upon inhibition of the processing protease by chelators. To determine whether the inhibition of the processing protease by R6G in intact cells could also result in an internalization of unprocessed pre-βF_1, the sensitivity of the precursor to externally added protease was tested. The pre-βF_1, but not the membrane-incorporated mature form, was completely digested by added proteinase K (fig.2). The precursor molecules present in the particulate fractions of both control and R6G-treated cells were also digested by the enzyme. Neither a prolonged chase with unlabeled methionine in the presence of R6G nor higher R6G concentrations (not shown) could render any

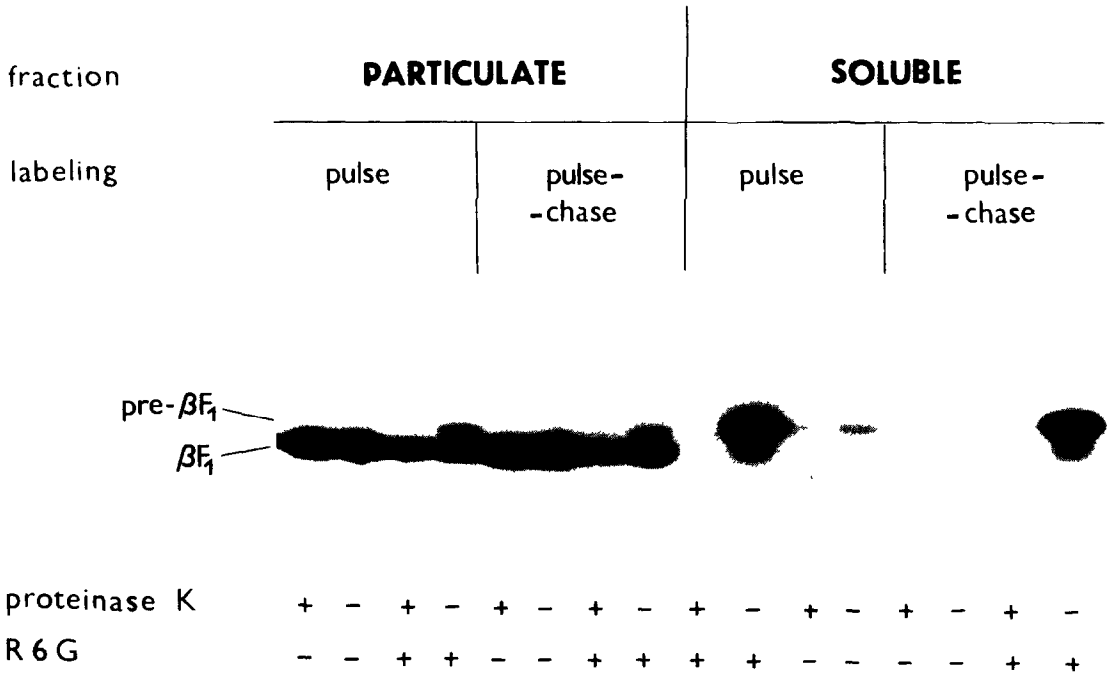

Fig.2. Sensitivity to externally added proteinase K of newly synthesized βF_1 in the cellular fractions of R6G-treated and control cells. The cells were labeled in the absence or presence of 0.65 μg R6G/mg protein and fractionated as described under fig.1. The soluble fractions were divided into two portions one of which received proteinase K (0.1 mg/ml). Both portions were kept for 30 min at 4°C. The particulate fraction was first resuspended as described in section 2, divided into two parts and treated with proteinase K as above. PMSF (2 mM) was added to stop the proteolysis and the samples were further processed and immunoadsorbed for βF_1 as described in fig.1. Lane on extreme right, [^{35}S]methionine-labeled mature βF_1.

significant portion of pre-βF_1 resistant to the externally added protease.

Similar experiments were performed with a mitochondrial protein, the import of which proceeds without proteolytic processing step but still requires a mitochondrial membrane potential [14]. Fig.3 shows that R6G neither increases the amount of the precursor of the ADP/ATP carrier in the cytosol fraction nor significantly inhibits its integration into the mitochondrial membrane. The lack of an effect of R6G on the distribution of the newly synthesized ADP/ATP carrier is in complete agreement with the proposed action of this inhibitor on the mitochondrial processing protease [6]. It also provides additional evidence that the inhibitory effect of R6G on the protein import is not due to uncoupling of oxidative phosphorylation. The described effects of R6G further illustrate the usefulness of this inhibitor for distinguishing between mitochondrial precursor proteins with and without a cleavable NH_2-terminal peptide extension.

Our results show that if the processing protease is inhibited in intact cells by R6G, the precursor of βF_1, which carries a cleavable NH_2-terminal extension, is accumulated in the cytosolic fraction of these cells. As detected by accessibility to externally added protease, the small portion of precursor molecules associated with the particulate membrane fraction is located outside the mitochondria. The massive accumulation in the cytosol of the precursor of βF_1 and the absence in the mitochondria of internalized precursor in R6G-treated cells indicate that in intact cells the proteolytic processing and the translocation of this precursor across mitochondrial membrane must be coupled at least to a certain degree. This is at variance with the conclusion drawn from in vitro studies that the proteolytic processing is not necessary for the translocation of precursor proteins through the

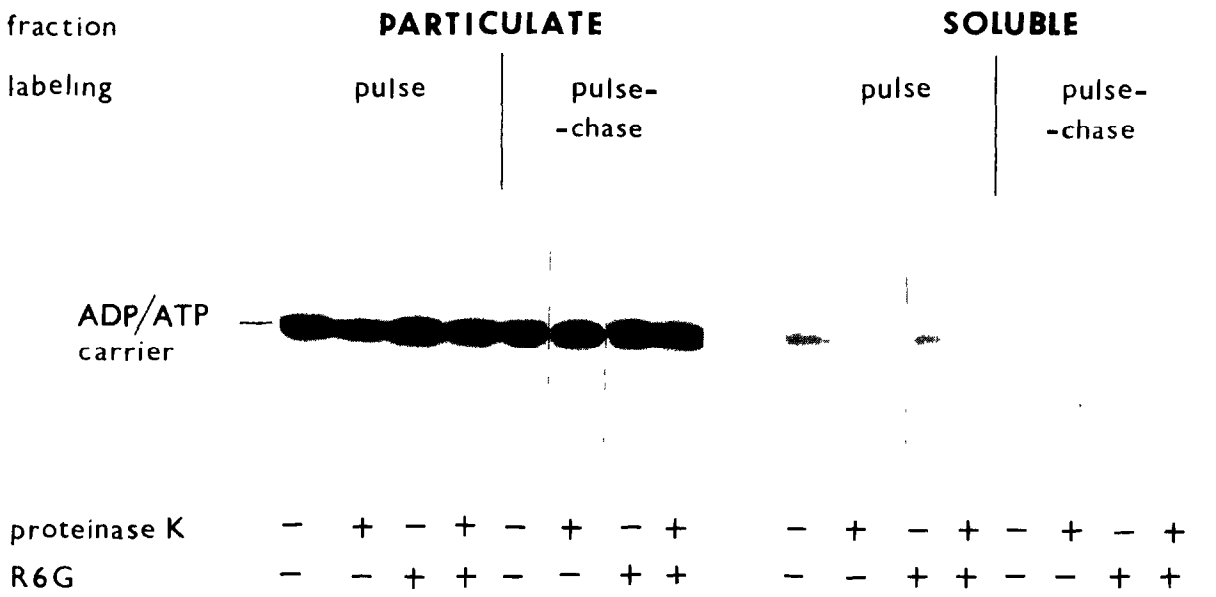

Fig.3. Sensitivity to externally added proteinase K of newly synthesized ADP/ATP carrier in the cellular fractions of R6G-treated and control cells. The cells were labeled, fractionated and treated with proteinase K as detailed under fig.2. The samples were immunoadsorbed for the ADP/ATP carrier.

mitochondrial membrane [5]. It is possible that coupling between membrane translocation and protein processing of the precursors might provide the cell with an additional regulatory step in biogenesis of mitochondria. This step may not be manifested in an in vitro system.

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