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CHANGES IN THE β-SUBUNIT OF MITOCHONDRIAL F_1ATPASE DURING NEUROGENESIS

Jean-Luc VAYSSIERE, Jean-Christophe LARCHER, François GROS, and Bernard CROIZAT

Laboratoire de Biochimie Cellulaire, Collège de France, Paris, France

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A polypeptide migrating in the area of the isotubulin in 2 D - gel electrophoresis of extracts from neuronal cells was characterized as the β-subunit of the F_1 ATPase matrix component. The synthesis of this subunit is enhanced during neurogenesis and the presence of an isoform was detected in adult mouse brain.

Using mouse neuroblastoma cells to study neurogenesis *in vitro*, we have analysed the changes in the distribution of cytoskeletal proteins in response to CCA, a potent neuroblastoma differentiation inducer (1). A large increase was observed in the synthesis of a polypeptide (protein 4) migrating in the area of the isotubulins on the 2 D - gel electrophoresis.

Various observations, in particular a significant increase in glucose utilization (2), had drawn our attention to a possible effect of CCA on mitochondrial metabolism and led us to examine the fate of mitochondrial (mit) proteins in the course of neuroblastoma differentiation (3, 4). In the present study, we identify the protein 4 as the β-subunit of the mitochondrial F_1 ATPase.

We have observed that the synthesis of the β-subunit is enhanced during the development of mouse cortical neurons in culture and brain maturation. Furthermore, we have detected the accumulation of an isoform in the adult brain. These changes may be discussed in relationship with neurogenesis.

MATERIALS AND METHODS

Materials

- Clone NIE 115 derives from mouse neuroblastoma C 1300. As previously described, cells were grown attached in Falcon culture dishes in a serum containing medium, and differentiated by addition of 0.1 % CCA or 2 % DMSO (5).

Abbreviations : CCA, 1 methyl cyclohexane sodium carboxylate ; DCCD, N-N'-dicyclohexylarcobodiimide ; DMSO, dimethylsulfoxide.

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- Primary cultures of neurons were established from fetal mouse brain cortex (15th day of gestation) as described by (6).

- Cortex mitochondria were prepared from 6 weeks old male mice (swiss clone).

- Nonactin was purchased from Sigma and [^{14}C] DCCD from C.E.A. (France). CCA was a gift from Sanofi Research Center.

Protein labeling and cell extract preparation

Control cells growing in a serum-containing medium, CCA- or DMSO-differentiated cells, and cells treated with nonactin were labeled for 5 h with [^{35}S] methionine (50 µCi / ml medium) prior to harvesting. The 10 cm diameter dishes were inoculated so that they contained 2.5 x 10^6 cells at the time of harvesting. Cell extracts were prepared and analysed on two-dimensional electrophoresis as in (7).

Mitochondria preparation

The method was described in (8). Neuroblastoma mitochondria preparation was adapted from Anderson (9), and cortex mitochondria from Nicholls (10).

F_0F_1 ATPase preparation

Submitochondrial fraction (brain cortex)

The separation of the outer membrane from mitoplasts was obtained after 20 min incubation in an hypotonic buffer (20 mM KH_2PO_4 , 0.02 % BSA pH 7.4).

Mitoplasts were then sonicated in a MSE sonicator (45 sec at 1.7 A). The technique described by (11) gave rise to four fractions, corresponding respectively to the matrix, the inner membrane, the intermembrane space and the outer membrane . The F_1 component was purified from the inner membrane by chloroform extraction according to (12).

[^{14}C] DCCD binding experiments

DCCD binding to the F_1ATPase component was processed as in (13).

RESULTS

Characterization of the F_0F_1 ATPase β-subunit

i) Using nonactin, mitochondrial function was impaired in neuroblastoma developing cells. The K^+ ionophore nonactin is known to alter the eletrochemical potential, thus preventing both the translocation of mit precursors into or across the mitochondrial membranes and their cleavage into mature polypeptides. Treatment of neuroblastoma cultures with 5 µM nonactin for 18 hours before harvesting resulted in the loss of the spot corresponding to protein 4 from the two-dimensional pattern of labeled polypeptides. A new spot appeared which is likely to correspond to the precursor of protein 4 (not shown here).

ii) Comparison of the electrophoregrams of extracts from neuroblastoma cells, primary cultures of neurons, mouse brain cortex and various tissues proves the ubiquity of protein 4. Its migration properties (apparent molecular weight : 52 kDa ; pH 5.8) (fig. 1) suggested it might be the F_1 matrix component of the mitochondrial ATPase, according to Hay et al. (14).

iii) In order to check this hypothesis, we have extracted, according to Beechey (11), the F_1 component from the submitochondrial fractions obtained from mouse brain cortex; the extract was analysed on SDS gel electrophoresis. The F_1 component has been reported to include several subunits (15) which we have localized on a 1 dimension gel, as shown on fig. 2 a. According to (16), the β-subunit carries the catalytic activity. The spot corresponding to the β-subunit is indicated on a 2 D - electrophoregram (fig. 2 b). It is strictly superimposable with protein 4.

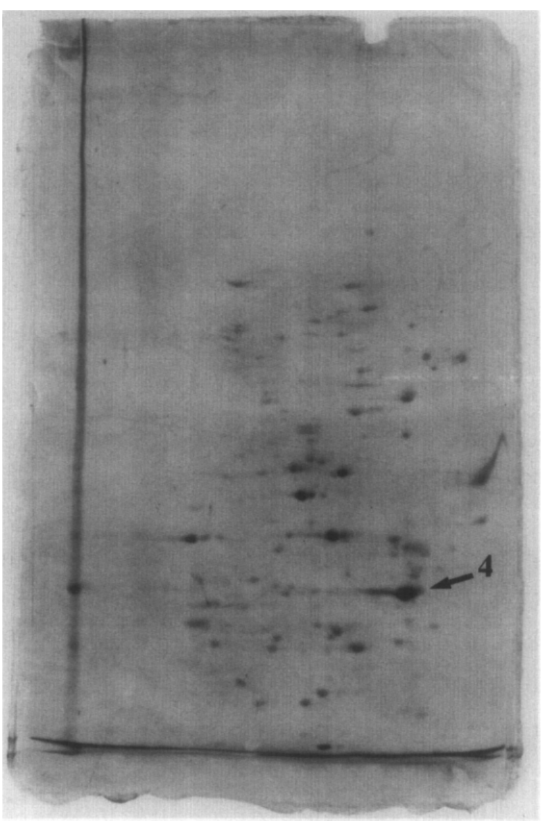

Fig. 1. *Two-dimensional SDS polyacrylamide gel of mouse brain cortex mitochondria.* Isoelectric focusing gel contained 0.4 % of pH 3.5-10 ampholines and 1.6 % of pH 5-8 ampholines. The SDS polyacrylamide gel for the second dimension contained 7.5 % acrylamide and 0.1 % bisacrylamide. Proteins are revealed by silver staining as in (19).

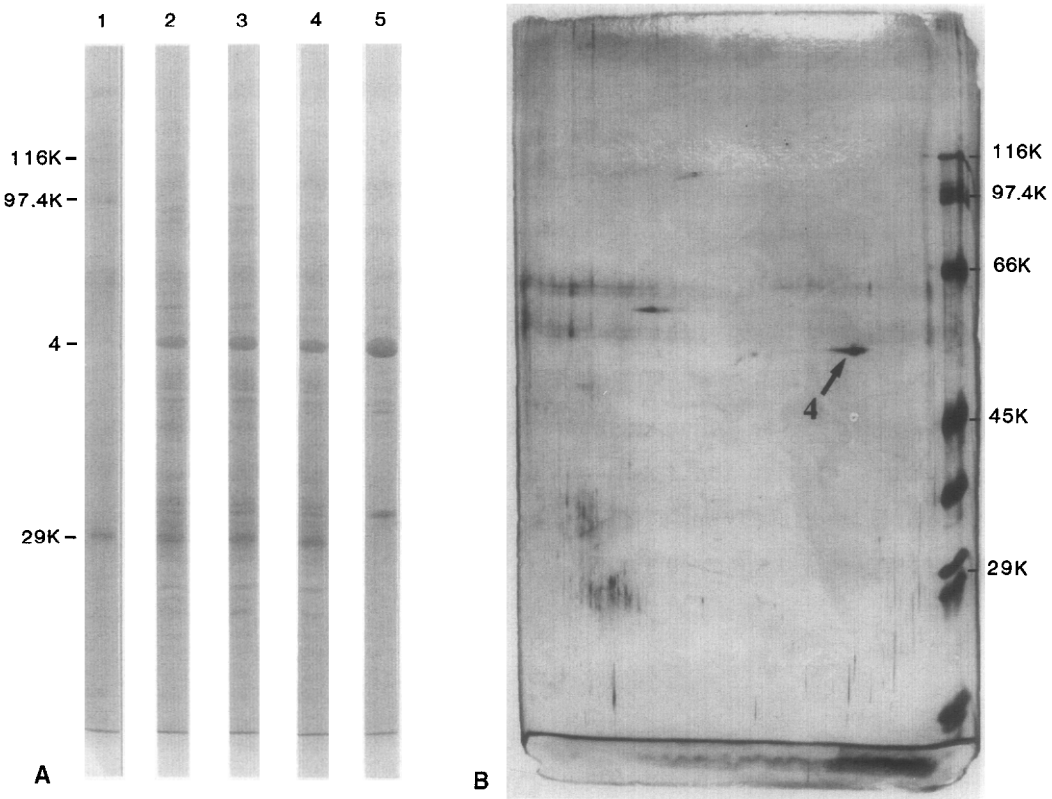

Fig. 2. *SDS gel electrophoresis of purified mitochondrial ATPase from mouse brain cortex.* a) One dimension slab gel electrophoresis. The proteins were analysed in linear polyacrylamide gradient as described by Laemmli (20), except for the acrylamide and bisacrylamide concentrations which were 12.5 % and 0.1 % respectively. 20 µg of proteins from each sample were layered on the gels, except for the ATPase (10 µg) and the molecular weight markers (5 µg). Lane 1 : molecular weight markers ; lane 2 : mouse brain cortex mitochondria ; lane 3 : mitoplast fraction ; lane 4 : mitochondrial inner membrane ; lane 5 : F_1 component of the mitochondrial ATPase. b) Two-dimensional SDS polyacrylamide gel. Isoelectrofocusing gel was as in fig. 1. The SDS polyacrylamide gel for the second dimension contained 12.5 % acrylamide and 0.1 % bisacrylamide.

iiii) The polypeptide corresponding to β-subunit can be characterized by its specific binding to DCCD. A covalent binding is formed with the molecule leading to the inhibition of the ATPase activity. Extracts from purified mitochondria previously incubated for 10 min in the presence of [^{14}C] DCCD were analysed on 2 D - gel electrophoresis. The corresponding autoradiograms revealed only one labeled spot superimposable with protein 4 (fig. 3).

Changes in the β-subunit during ontogenesis

i) *Quantitative changes*

- Neuroblastoma cultures -

[^{35}S] methionine incorporation into β-subunit was compared in growing

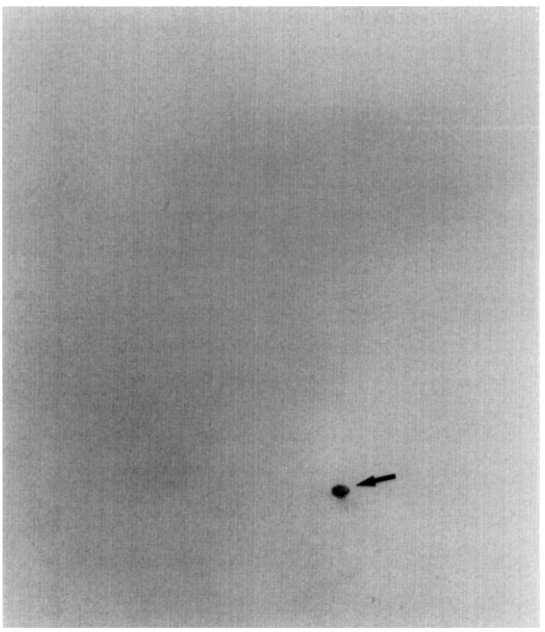

Fig. 3. *[^{14}C] DCCD binding to mitochondrial ATPase β-subunit.* Extracts from purified mitochondria were pre-incubated for 10 min in the presence of 10 μCi of [^{14}C] DCCD per mg of protein. They were then analysed on 2 D - gel electrophoresis as in fig.1. Fig.3 shows the autoradiogram corresponding to the electrophoregram. The arrow indicated the labeled spot superimposable to protein 4.

neuroblastoma and after induction by CCA or DMSO, two drugs known to cause morphological differentiation. The data are expressed relative to the value relative rate of methionine incorporation was 1.5. After 7 days in presence of DMSO we found a twofold enhancement.

- Primary cultures of neurons -

[^{35}S] methionine incorporation was enhanced during *in vitro* differentiation of neurons from fetal mouse cortex. The value found after 36 hours of culture is made equal to 1. After 8 days, 15 days, and 21 days, relative rates of methionine incorporation were 1, 1.25 and 2 respectively.

- Mouse brain cortex -

The distribution pattern of proteins on 2 D-electrophoregrams from fetal and adult cortex shows a relative accumulation of the β-subunit in the adult (fig. 4).

ii) *Qualitative changes*

- Apparition of an isoform -

Using different conditions of electrophoresis (see legend of fig. 5), an

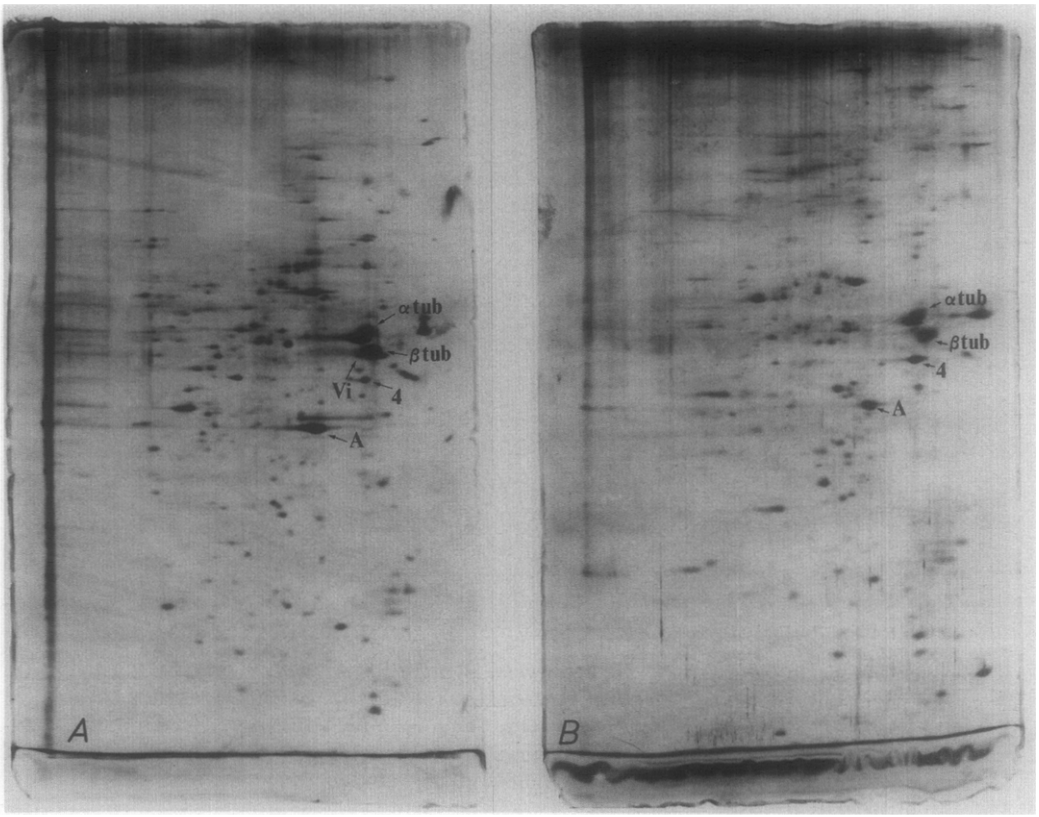

Fig. 4. *Evolution of mitochondrial ATPase β-subunit during mouse brain cortex maturation.* Extracts were analysed on two-dimensional gel electrophoresis as described in fig. 2 b. a) Extracts from brain cortex at the 16th day of fetal life ; b) Extracts from adult brain cortex. 80 mg of proteins were layered on both gels. α tub : α-tubulins; β tub : β-isotubulins ; A : actin ; Vi : vimentin.

acidic isoform of β-subunit showing the same molecular weight was detected on the electrophoregrams of mit proteins from adult mouse cortex (fig. 5 a). In contrast, the spot corresponding to the isoform was very weak in the extracts from fetal and neonatal cortex (fig. 5 b), and from neuron cultures (fig. 5 c). It was not detected in the neuroblastoma (not shown here).

The acidic form, named a, was characterized by peptide analysis. Alternatively, the ubiquitous basic form b, the acidic form a, and a mixture of both forms gave rise to the same pattern of peptides (fig. 6). [^{14}C] DCCD does not bind to isoform a (fig. 5d).

DISCUSSION

We report here evidence to characterize a major polypeptide migrating in the area of the isotubulins on the 2 D-electrophoregrams. This polypeptide,

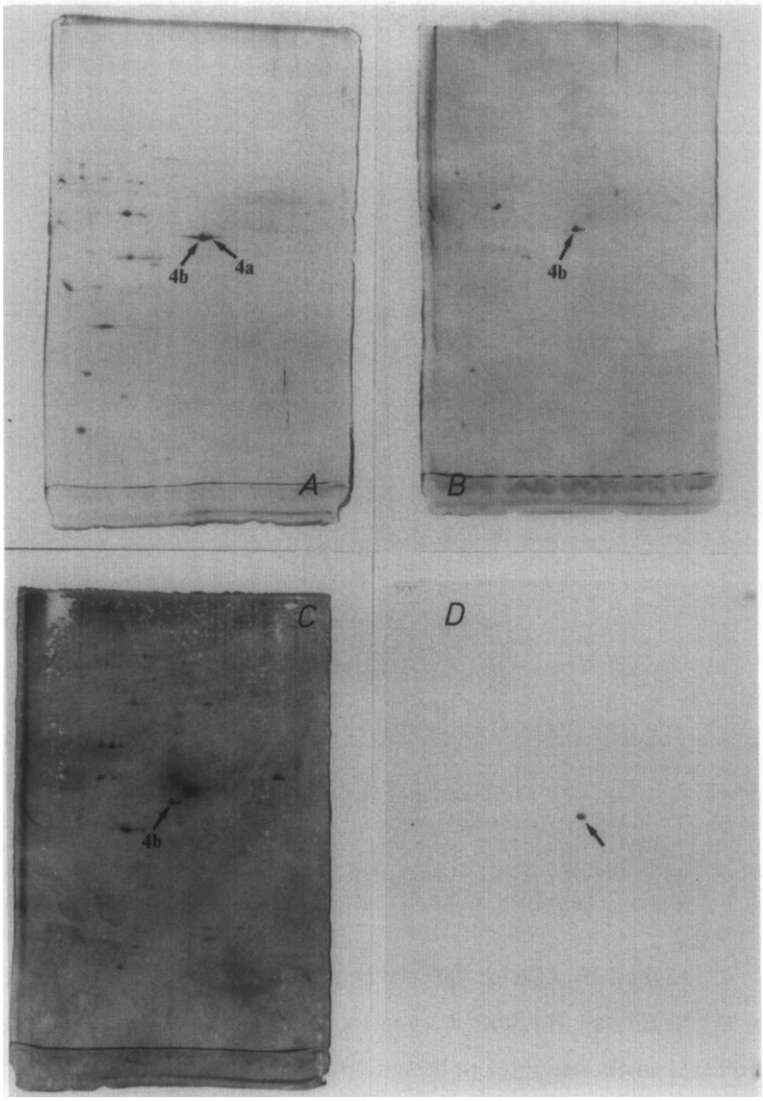

Fig. 5. *Comparative analyse of the mitochondrial ATPase β-subunit.* Extracts were analysed on two-dimensional gel electrophoresis as described in fig. 4, except as following : isoelectric focusing contained 2 % ampholines of pH 4-6. a) 4a corresponds to the acidic isoform, and 4b to the ubiquitous isoform. Mitochondria from adult brain cortex ; b) mitochondria from fetal brain cortex ; c) mitochondria from differentiated cortical neurons in culture ; d) autoradiogram corresponding to the electrophoresis of mitochondria from adult brain cortex after a 10 min incubation with 10 µCi of [^{14}C] DCCD per mg of protein. The arrow indicates the labeled spot superimposable to ubiquitous isoform b from adult brain cortex.

which had been described as a β-isotubulin (17) is, in fact, the β-subunit of the F_1 matrix component of the F_0 F_1 ATPase.

We have observed an approximatively twofold enhancement in the [^{35}S] methionine incorporation into the β-subunit during neuroblastoma

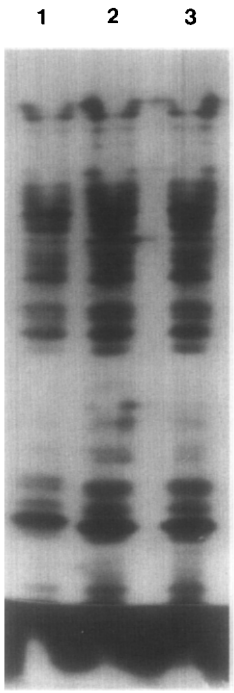

Fig. 6. *Comparative maps of the isoforms of the β-subunit ATPase.* Peptide analysis was carried out according to (21). Sections containing a and b isoforms were cut out from two-dimensional SDS gels overlaid with 20 mg of protease from *Staphylococcus aureus V 8* diluted in Tris 0.125 M pH 6.8, 0.1 % SDS, EDTA 1 mM, 10 % glycerol. Digestion proceeded directly in the stacking gel during the subsequent electrophoresis. Lane 1 : acidic isoform a ; lane 2 : mixture of both isoforms ; lane 3 : ubiquitous isoform b.

differentiation induced by CCA or DMSO, and the *in vitro* development of non-treated cortex neurons. There is a specific increase in the β-subunit synthesis relatively to total mitochondrial proteins (not reported here). The β-subunit accumulation, also observed during brain maturation, is partly due to the dramatic increase of the acidic isoform in the adult. This result is not surprising, considering that the successive steps of neurogenesis can be grossly summarized as a) arrest of cell division, b) morphogenesis, c) biochemical differentiation, d) synaptogenesis, and e) for the brain, multiplication followed by terminal differentiation of glial cells. This process not only requires energy but also various regulations, under the control of the mitochondrion, at the level of the cellular pH and the ionic fluxes (18).

The accumulation of an isozyme of the β-subunit in the adult nervous tissue deserves further discussion. The acidic form can be considered as a late marker of differentiation. There is no doubt concerning its characterization since peptidic analysis reveals its identity to the ubiquitous basic isoform, and it copurifies with the matrix F_1 ATPase component. However, we have observed

that it does not bind to DCCD (or binds very little), and we cannot suggest any specific role or function.

One can suggest various hypothesis to account for the absence of the acidic form in neuroblastoma cultures : i) neoplasic clones do generally express embryonic genes. Contrastly, the isoforms corresponding to late markers of differentiation are frequently repressed ; ii) moreover, C 1300 neuroblastoma derives from the neural crest which appears at a very early stage of the embryogenesis. Despite the acquisition of many morphological, biochemical and electrophysiological properties in neuroblastoma treated cells, the neurogenesis cannot be assumed as totally complete. In particular, some late markers may remain not expressed.

This last hypothesis, concerning the kinetics of appearance of differentiation markers, can be proposed for primary cultures of neurons provided one assumes that *in vitro* spontaneous development grossly mimics normal *in vivo* maturation, as far as the chronology is concerned. In particular, neurons were obtained from mouse cortex at the 15th day of fetal corresponds approximatly to the 15th day of neonatal life, a phase where the acidic form is also barely detected *in vivo*.

In conclusion, the quantitative and qualitative changes observed in the β-subunit - as in various other mit-markers (not reported here) - are in agreement with the hypothesis that the neural tissue ontogenesis is accompanied by a maturation of the mitochondrion ; the organelle forms a dynamic structure changing according to its cellular environment.

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