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CHANGES IN MITOCHONDRIAL PROTEINS DURING NEUROBLASTOMA DIFFERENTIATION

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SUMMARY. The evolution of three major mit-proteins was followed in neuroblastoma cells cultured in different conditions of differentiation. 1 methyl cyclohexane carboxylic acid (CCA) was found to stimulate the synthesis of the three mit-protein markers. This result, compared to the effects of oligomycin, an inhibitor of mitochondrial function, favours the hypothesis that CCA induces *in vitro* neurogenesis through a general metabolic alteration.

Most of our information concerning brain metabolism emphazises the high level of energy metabolism and oxygen consumption. The availability of clonal cell lines of neuronal origin and of primary cultures has made it possible to compare the energy metabolism of homogeneous cell populations with that of the mixed cell population in the brain. Studies confirm that O_2 consumption of neurons grown in culture is always higher than glial respiration (1). Furthermore, the evolution of enzymatic activities in cell culture indicate that differential modifications of the energy metabolism occur in neuronal and glial elements (2). Neuronal maturation reflects a shift of the energy metabolism towards a more aerobic form (2, 3). Alternatively, the perfusion of anaesthetics in brain induces a reduction of neuronal activity as well as of energy metabolism, oxygen consumption and transmitter turnover (4, 5).

These considerations led us to further study the effects on mouse neuroblastoma cells of a pharmacologically active substance, known for its antianoxic effects on the rat brain (6) and which we had shown previously to act as a potent neural differentiation inducer, the compound 1 methyl cyclohexane carboxylic acid (CCA).

CCA promotes a significant increase in the level of cellular energy metabolism in developing neuroblastoma in tissue culture conditions. This increase is estimated by the cellular accumulation of the complex between the phosphorylated [^{14}C]deoxyglucose and hexose phosphate isomerase, a phenomenon which reflects glucose utilization, according to

Abbreviations: mit-protein, mitochondrial protein; mitpro, precursor of mitochondrial protein.

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Sokoloff (7). A considerably higher amount of radio-activity - 2.5 to 4 times - was found in CCA-treated cells, as compared to control, undifferentiated cultures or to neuroblastoma induced by other treatments, corresponding to a higher rate of deoxyglucose penetration and utilization (8). This "burst" in cellular energy metabolism occurs prior to any visible change in cell growth and morphology. It was thus postulated that it could play a role in triggering the neuronal differentiation. It is interesting to note that the drastic changes in glucose utilization induced by CCA are specific of neuroblastoma cells and not found in other systems, such as myoblast cultures.

These observations have drawn our attention to a possible general relationship between mitochondrial function and neurogenesis, and led us to examine more particularly the fate of mitochondrial (mit) proteins in the course of neuroblastoma differentiation. Few data concerning the role of mitochondria in neuronal function are available. Studies, reporting the effects of anaesthetics on electroencephalogram, provide evidence for the involvement of mitochondrial enzymes in the relationship between cerebral metabolism and function (3, 9, 10, 11). Ultrastructural studies in the rat brain also emphasize the fact that the number and morphology of mitochondria undergo important changes in active neuronal cells (12).

As an approach to this study we have first attempted to characterize some mit-protein markers in C-1300 neuroblastoma (N1E-115 clone). Those selected in this study have also been detected in the mouse brain tissue. The present paper indicates that CCA, and to a much lesser extent DMSO but not serum deprivation, strongly stimulates the synthesis of mit-proteins supporting the view that the strong CCA inducer effect could be correlated with an interaction at the mitochondrial level. Brain maturation is accompanied by an increased synthesis of mit-protein.

MATERIALS AND METHODS

Cell Culture

The N1E-115 and NS-20 clones from mouse neuroblastoma C-1300 were used. Culture conditions have been described in (13). Morphological differentiation was induced either by withdrawing serum from the culture medium or by addition, to a serum containing medium, of 0.1 % CCA for 3 days or 2 % DMSO for 7 days. Cultures were changed with fresh medium every 24 hours. Mitochondrial function was impaired by adding 0.6 µM oligomycin to the culture medium or 5 µM nonactin (Sigma) for 18 hours prior to harvesting.

Protein labeling and cell extracts

Cells were labeled for 6 or 24 hours with [^{35}S]methionine (25 µCi / ml medium) prior to harvesting. The cells extracts were prepared and analysed on 2D-electrophoresis as described in (14).

Preparation of mit-proteins

Purification of mit-proteins was achieved according to Anderson (15). Cells prelabeled with |^{35}S|methionine were homogenized in 0.25 M sucrose / 0.3 mM EDTA, pH 7.4, and spun at 600 x *g* for 10 min. The supernatant was centrifuged at 100,000 x *g* for 10 min, and the resulting pellet was washed twice. The last pellet was resuspended in 50 % (wt/wt) sucrose / 0.3 mM EDTA and layered under a step gradient of 44.5 % and 41 % sucrose. This was centrifuged for 2 h at 45,000 rpm in a Beckman type 50 rotor. The material banding between 41 % and 44.5 % sucrose was diluted into 0.25 M sucrose / 0.3 M EDTA, centrifuged at 10,000 x *g* for 10 min and the resulting pellet was dissolved, for protein analysis, in 8 M urea / 20 % Nonidet P-40 / 2 % ampholines (LKB, 3.5 - 10) / 1 % mercaptoethanol.

Peptide analysis

Peptide analysis was carried out according to Cleveland *et al.* (16). Sections containing mit-proteins were cut out from two-dimensional SDS gels overlaid with 20 ng of protease from *Staphylococcus aureus* V8 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.

RESULTS

Characterization of some neuroblastoma mitochondrial proteins

Two experimental procedures were used in order to identify mit-proteins in neuroblastoma cells : i) purified mit-proteins were prepared as described in the "Materials and Methods" and analysed on two-dimensional electrophoresis ; ii) mitochondrial function was impaired in whole developing cells using two specific inhibitors, nonactin and oligomycin. The K^+ ionophore, nonactin, is known to alter the electrochemical potential, thus preventing both the translocation of mit-precursors into or across the mitochondrial membranes and their cleavage into mature polypeptides. Conversely, the addition to culture medium of oligomycin, an inhibitor of phosphorylative oxydations, was shown, in the present work, to enhance methionine incorporation into the mit-proteins, whose precursors are not processed in the presence of nonactin. The combination of these procedures allowed us to select three mit-protein-markers (called 4, 5 and C_1 respectively). They appear as major spots on the electrophoregram of purified neuroblastoma mitochondria, after appropriate lysis, as shown on Fig. 1. Fig. 2a and 2b show the electrophoregrams of total cell proteins from a) control cultures : arrows indicate positions of mature proteins 4, 5 and C_1 ; b) nonactin-treated cultures : arrows point towards the virtual positions of the corresponding proteins, which have disappeared.

Treatment of cultures with 5 µM nonactin for 18 hours before harvesting resulted in the loss of the spot corresponding to protein 4 (apparent molecular weight : 52 kda ; pH_i 5.8) from the two-dimensional pattern of labeled polypeptides. A new spot appeared which is likely to correspond to the precursor (mitpro) of protein 4 (compare Fig. 2a to 2b).

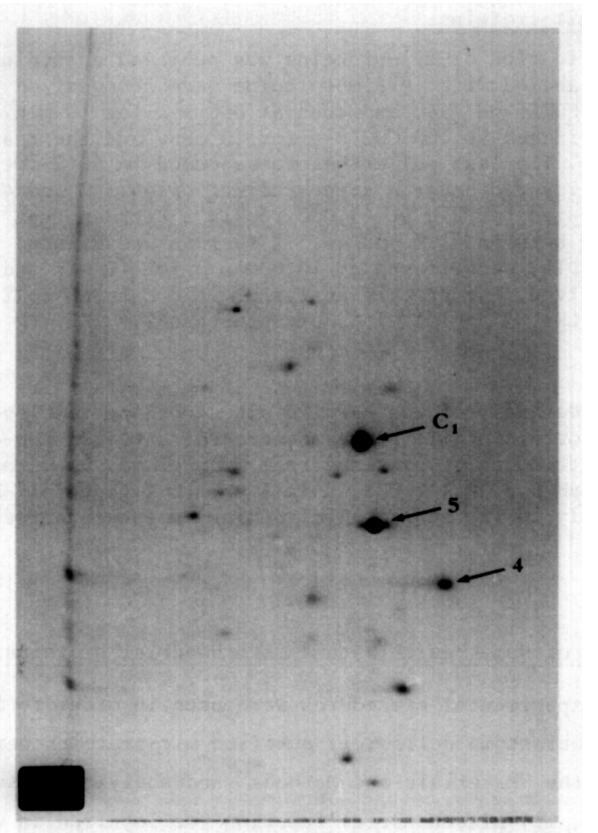

Fig. 1 Electrophoregram showing mit-proteins in a preparation from N1E-115 neuroblastoma cells grown in a complete medium (DMEM + 7.5 % fetal calf serum) and labeled during 6 hours prior to harvesting. An amount of radioactivity corresponding to 2.10^5 cts / min of ^{35}S-labeled proteins was loaded on the gel.

The precursor is made by the nucleo-cytoplasmic genetic system (17). After 24 hours labeling prior to harvesting, methionine incorporation into protein 4 from oligomycin-treated culture increased 1.5 fold relative to the value found in control culture. Electrophoretic characteristics of this protein suggest that it could be the β subunit of F_1 ATPase (data not shown).

The spot corresponding to protein C_1 (apparent molecular weight : 68 kda ; pH_i 5.8) in total extracts from methionine labeled control cultures disappears upon pretreatment with nonactin. However, we were unable to locate a putative precursor. (The latter might be present on the gel with important molecular weight or(and) pH modifications). Methionine incorporation into protein C_1 after oligomycin treatment increased 2.7 fold.

The synthesis of protein 5 (apparent molecular weight : kda ; pH_i 5.5) was also inhibited by nonactin in neuroblastoma cells and like in the case for protein C_1 no candidate for a mitpro was identified on the electrophoregram. Methionine incorporation after oligomycin treatment was

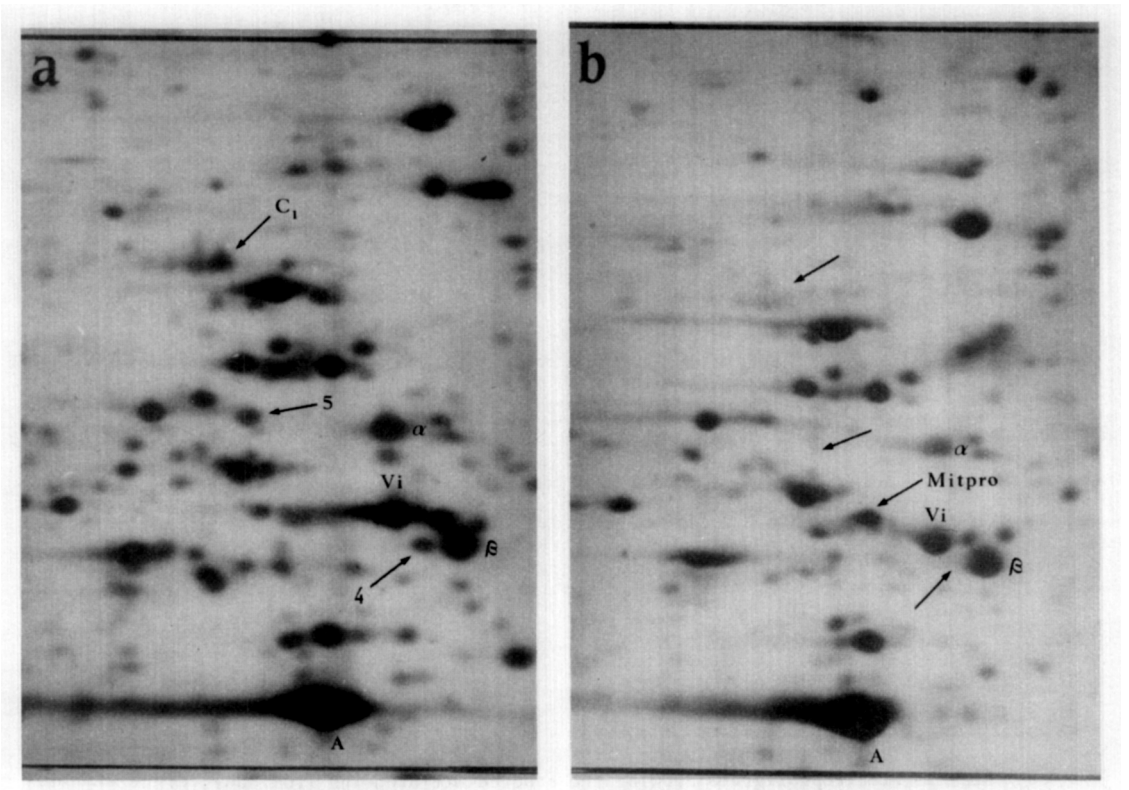

Fig. 2 Areas from two-dimensional gels loaded with NIE-115 neuroblastoma extracts. Mature mit-proteins or the precursor of mit-protein 4 (mitpro) are indicated by arrows. α and β isotubulins (α, β), vimentin (Vi) and actin (A) are shown as reference markers. Cells were labeled for 24 hours prior to harvesting. 7.10^5 cts / min corresponding to ^{35}S-labeled proteins were loaded on each gel. a) Control cells growing in a complete medium. b) Nonactin-treated cultures. The drug was added 18 hours prior to harvesting. Arrows indicate the virtual positions of the mit-proteins which have disappeared.

1.3 fold greater than in control cells. The electrophoretic characteristics of protein 5 suggest that it may be identical to the IEF 24 protein already described by Larsen *et al.* (18) which, according to these authors, would play the role of a linker, connecting the mitochondria to intermediate filaments structures. The identification of protein 5 is in progress.

Detection of the mitochondrial proteins in fetal and adult mouse brain

Proteins 4, 5 and C_1 are readily detected by 2D-electrophoresis of an extract from fetal mouse brain (Fig. 3). Identification of these proteins was made by comparing the protease digestion products from neuroblastoma and brain mit-proteins, as shown in Fig. 4.

Methionine incorporation into mit-proteins was enhanced during mouse brain maturation, as shown by the examination of the autoradiograms from adult and fetal brains (see legend to Fig. 3).

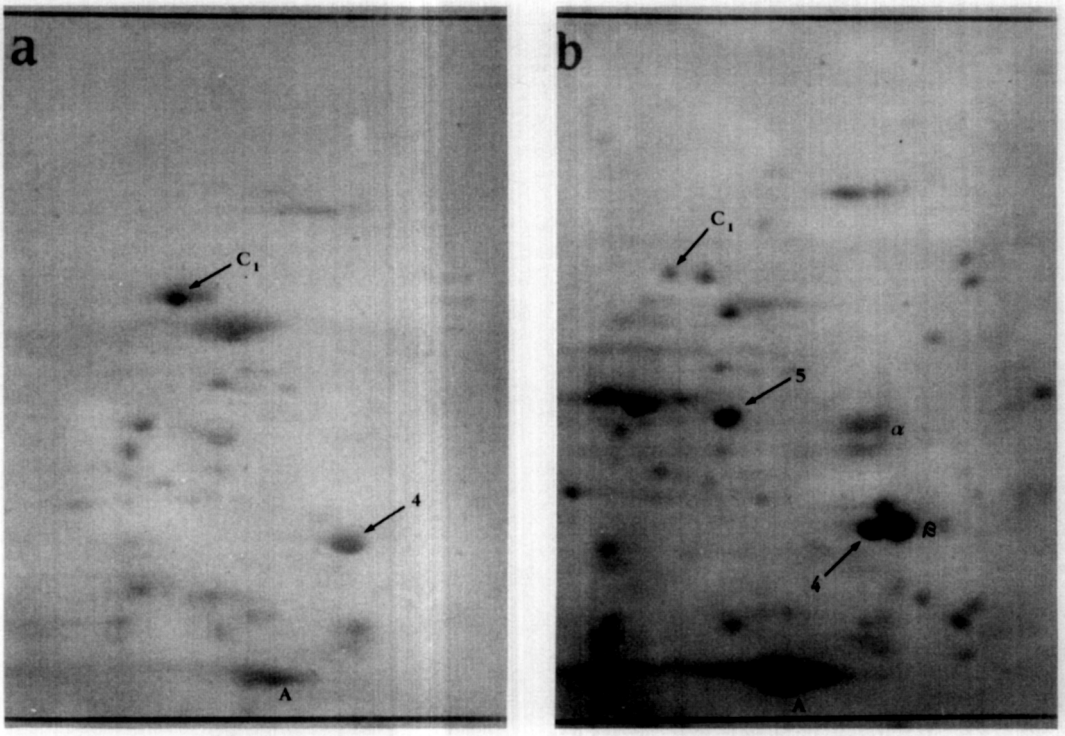

Fig. 3 Electrophoregrams showing the studied mit-proteins from adult (a) and fetal (b) mouse brain. Only the relative intensities of the spots on each electrophoregram must be taken into consideration. a gel was loaded with 3.10^5 cts / min corresponding to ^{35}S-labeled proteins while b gel was loaded with 10^6 cts / min. This is visualised by a weak spot corresponding to actin and no detectable isotubulin on a gel. Contrastly, the spots corresponding to mit-proteins C_1 and 4 appeared as major ones, thus indicating an important enhancement of three proteins at the adult stage.

Quantitative changes in the mit-proteins during neuroblastoma differentiation

$[^{35}S]$methionine incorporation into mit-proteins 4, 5 and C_1 was compared in growing neuroblastoma and after induction by CCA or DMSO, two drugs known to cause morphological differentiation. The data are expressed relative to the values found in control culture made equal to 1. After 3 days of CCA treatment, relative rates of methionine incorporation were 1.5, 1.4 and 1.9 for proteins 4, 5 and C_1 respectively. After 7 days in presence of DMSO we found a two fold enhancement for protein 4 but no change for proteins 5 and C_1. No change was observed either when differentiation was elicited by serum withdrawal.

DISCUSSION

We report preliminary results concerning the comparable evolution of three major mit-proteins (4, 5 and C_1) during neurogenesis. Protein 5

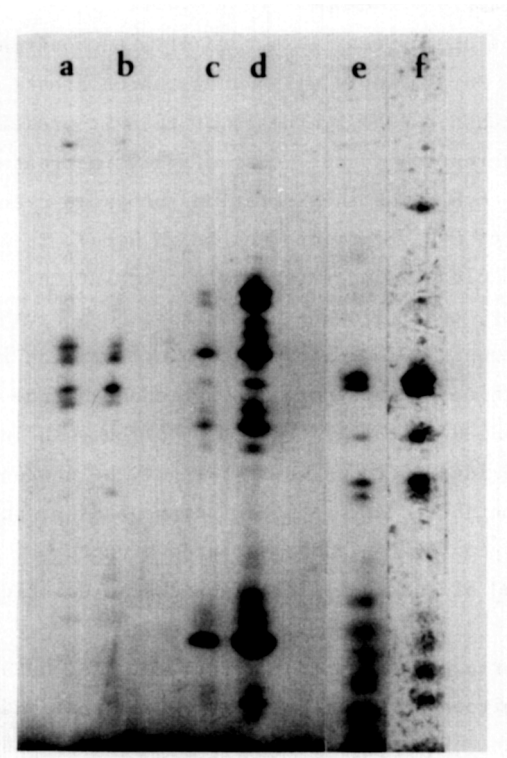

Fig. 4 Comparative peptide maps of mit-proteins from mouse brain and from neuroblastoma. a) mit-protein 5 from neuroblastoma, b) same from mouse brain. c) mit-protein 4 from neuroblastoma, d) same from mouse brain. e) mit-protein C_1 from neuroblastoma, f) same from mouse brain.

is likely to be identical to protein IEF_{24} already described by Larsen *et al.* (18). In brain, like in *in vitro* developing neuroblastoma, neurogenesis is accompanied by a marked increase in the synthesis of the three mit-proteins. Except for C_1 which we have only found so far in mouse brain and neuroblastoma, proteins 4 and 5 are probably ubiquitous, since they have been detected in mouse muscle, liver and kidney (data not shown). The possibility that they might undergo important developmental changes in other tissues than brain is presently under investigation.

Using CCA, a very active inducer of *in vitro* neuroblastoma differentiation, our attention had previously been called on its possible action at a mitochondrial level. Accordingly this agent, which causes ultimate modifications in the synthesis of membrane bound and the cyto-skeletal proteins (14, 19, 20), also greatly alters the cellular energy metabolism as measured by glucose utilization. In the present paper, an analysis of purified neuroblastoma mit-proteins has allowed us to propose that proteins 4, 5 and C_1 could act as major sites for a developmentally controlled modification, since methionine incorporation into these markers varies during the course of CCA-induced neurogenesis.

The effect of various inhibitors of mitochondrial functions has also been analyzed. Surprisingly oligomycin, which blocks the oxydative phosphorylations, enhances the synthesis of the mit-protein markers, like does CCA. It is interesting to note that oligomycin-treated cells exhibit an increase in the methionine incorporation into some cytoskeletal components like after CCA treatment (not shown here). It is not yet possible however to clearly understand this correlation.

At present, the following points have been established during CCA induced neurogenesis : i) 2-Deoxyglucose utilization is markedly increased ; ii) methionine incorporation is enhanced into all the mit-protein-markers studied. With the effects of oligomycin in mind, these results support the idea that CCA would trigger the neuronal differentiation through a general metabolic alteration causing perhaps, subsequently, rearrangements in cytoskeleton and associated structures. This would end up in morphological and biochemical phenotypes characteristic of mature neurons.

The differences between the effects of CCA, DMSO and serum withdrawal on the mitochondria emphasize the idea that "neuroblastoma differentiation" can correspond to different series of inducing events. Several paths may lead to a final state of morphologically differentiated cell.

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