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Differentiation of neuroblastoma cells correlates with an altered splicing pattern of tau RNA

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Morphological differentiation of N2A neuroblastoma cells is associated with an altered splicing of the gene of the microtubule-associated protein, tau. Two populations of RNA (coding for tau proteins containing three or four tubulin-binding motifs) are present in a similar proportion in undifferentiated neuroblastoma cells while in differentiated cells the proportion is changed in favour of that population coding for tau protein containing four tubulin-binding motifs. An increase in a high molecular weight tau isoforms correlates with the increase in the RNA population coding for four tubulin-binding motifs. A possible consequence of expressing a higher proportion of the tau protein containing four tubulin-binding motifs could be an increase in microtubule stability of differentiated neuroblastoma cells.

Tau protein; Microtubule stability; PCR

1. INTRODUCTION

The extension of neutral processes is an event dependent on microtubule assembly [1,2]. Kirschner and Mitchison suggested that such extension takes place due to stabilization of a microtubule population upon the reception of an external signal [3]. Thus, the molecules which can stabilize microtubules have been studied. These molecules have been characterized in the same way as the proteins that associate to brain microtubules (MAPs) [4]. Tau, one of these MAPs [5,6], has been specifically involved in the stabilization of microtubules in vivo [7] and in neurite extension [8]. Brain tau protein is a family of related peptides [6] which are translated from different mRNAs, generated by alternative splicing of a precursor RNA transcripted from a single gene [9–11]. Brain tau peptides can be divided in two classes, one containing three repeated sequences involved in the binding of tau to tubulin, and a second containing four of those sequences [9,12–15]. The first class is mainly present in early developmental stages, while the second is mainly expressed in mature brain [12–14]. It has been suggested that tau containing four tubulin-binding motifs associates to (and stabilizes) microtubules better than tau containing three motifs [16].

Neuroblastoma cells have been used as a model to study the extension of neural processes. Under certain conditions, such as the removal of serum from culture medium, neuroblastoma cells extend neurites [17]. It has been suggested, that microtubule proteins play a role in such neurite extension [1,2]. During this event a net microtubule assembly takes place, probably depedent, at least in part, on the modification (phosphorylation) of microtubule proteins [18–21] like tubulin and MAP_1B. However, a possible role for other proteins in increasing the amount of microtubule polymer can not be ruled out. One of these proteins could be tau, as previously indicated. The presence of tau-related proteins in neuroblastoma cells has been described [22] although some of their characteristics differ from those of brain tau peptides. In neuroblastoma cells peptides with a similar electrophoretic mobility to tau were observed [22,23] together with a tau-related protein with a higher apparent molecular weight of 120 kDa, mainly present in differentiated cells [22]. A similar protein was found in pheochromocytoma (PC 12) cells [22,24]. This protein appears to be specific for cells from peripheral nervous systems [25].

In this work we have analyzed the role of tau-related proteins during the morphological differentiation (neurite extension) of neuroblastoma cells. Our results indicate that an increase in the expression of tau-related peptides containing four tubulin-binding motifs accompanies differentiation of neuroblastoma cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Monoclonal tau antibodies, 7.51 and 133, have been described elsewhere [26,27]. Polyclonal tau antibodies have been also described [27].

Oligonucleotides spanning tau cDNA position 573–592 (R1), 942–966 (R2) and 1133–1152 (Ct), were synthetized using a DNA synthetizer (Applied Biosystems Inc.). Restriction nucleases *Hin*dIII and *Sma*I were purchased from Boehringer-Mannheim.

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2.2. *Cell culture*

N2A mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and induced to differentiate by transfer to DMEM without serum. Phosphate labelling was performed for 20 h with 1 mCi/ml ^{32}P (Amersham) as previously described [20]. [^{35}S]Methionine labelling was done for 16 h with 0.5 Ci/ml (Amersham).

2.3. *Immunofluorescence analyses*

Cell cultures were grown, fixed and processed as previously indicated [17,28]. The samples were examined using a confocal fluorescence microscope.

2.4. *RNA isolation and cDNA synthesis*

RNA was prepared by the method of Han et al. [29]. cDNA synthesis was carried out by doing a further addition of AMV reverse transcriptase (5 U/μg) and 200 mM of each of the four deoxynucleotide triphosphates. After synthesis the RNA was degraded by RNase A.

Oligonucleotides R1 and Ct or R1 and R2 were mixed with total cDNA. For PCR amplification the cDNA products were mixed with 1 μM of the oligonucleotides used. Two pair of primers were used for each analysis, (see Fig. 4), R1 and Ct, and R1 and R2. The obtained cDNA was characterized by gel electrophoresis, Southern blot [30] and by partial digestion with endonuclease restriction enzymes.

2.5. *Protein preparation*

Total protein or heat-resistant protein from neuroblastoma cells was obtained by homogenization of the cells with a teflon–glass homogenizer and centrifugation of the cell homogenates in a Beckman airfuge (2 min at maximum speed). The heat-resistant fraction was obtained upon boiling of the sample for 5 min in the conditions described by Herzog and Weber [31]. The perchloric-soluble, glycerol-insoluble protein was isolated as described by Lindwall and Cole [32].

Proteins were characterized by electrophoresis on polyacrylamide gels [33] and identified by Western blot [34].

3. RESULTS

3.1. *Localization of tau related proteins in neuroblastoma cells*

N2A neuroblastoma cells were cultured in the presence (undifferentiated cells) or in the absence (differentiated cells) of serum [20] and fixed with cold methanol for immunofluorescence analyses. The analyses were carried out using different antibodies againt tau. An example, using one of them, is indicated in Fig. 1, in which a morphologically differentiated (i.e. a cell with extended processes) is shown together with an undifferentiated, spherical, cell. The antigen reacting with tau antibody is localized to cytoskeletal structures identified as microtubules, since these structures are not present in colcemide-treated cells (not shown). Also, as indicated, the presence of tau-related protein is evident in the extended neuronal processes in morphologically differentiated cells.

3.2. *Characterization of tau related proteins in neuroblastoma cells*

To isolate tau-related proteins from undifferentiated and differentiated neuroblastoma cells the protocol of Lindwall and Cole [32] for brain tau was followed. The method is based in the solubility of tau in perchloric acid and its precipitation by glycerol [32]. Fig. 2 shows the polypeptide pattern of the protein fraction which is soluble in perchloric acid and insoluble in glycerol, obtained from undifferentiated or differentiated neuro-

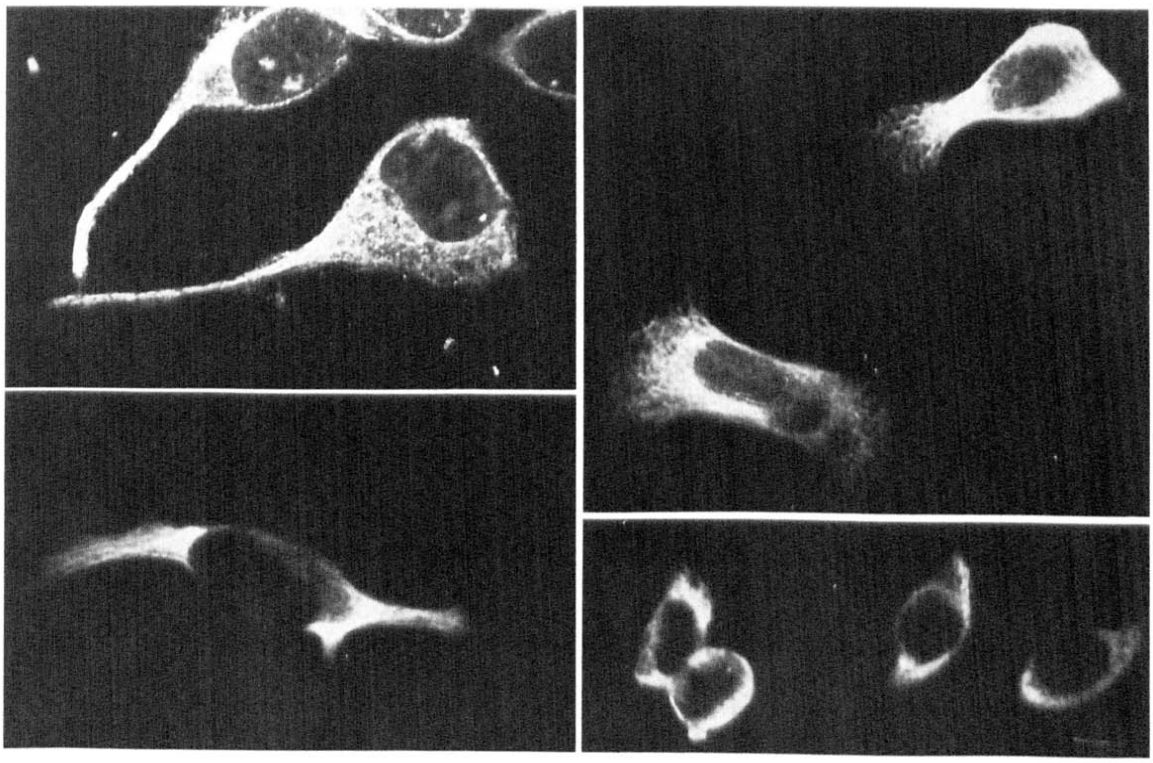

Fig. 1. Immunofluorescence staining pattern of neuroblastoma cells incubated with a polyclonal antibody to tau. Morphologically, two types of cells can be observed, undifferentiated (spherical shape) and differentiated (asymmetric shape).

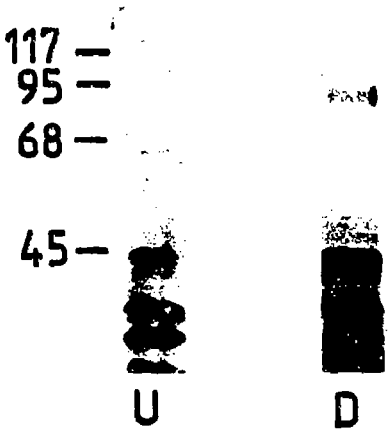

Fig. 2. Characterization of perchloric-soluble, glycerol-insoluble, proteins from neuroblastoma cells. N2A neuroblastoma cells were grown in the presence of 0.5 mCi/ml of [^{35}S]methionine and in the presence (U) or the absence (D) of serum. The cells were homogenized as indicated in section 2 and 2.5% (v/v) perchloric acid was added. The soluble fraction after centrifugation (15,000 × *g* for 15 min) was mixed with 25% glycerol and the pelleted protein, after centrifugation (1,500 × *g* for 15 min), was characterized by electrophoresis.

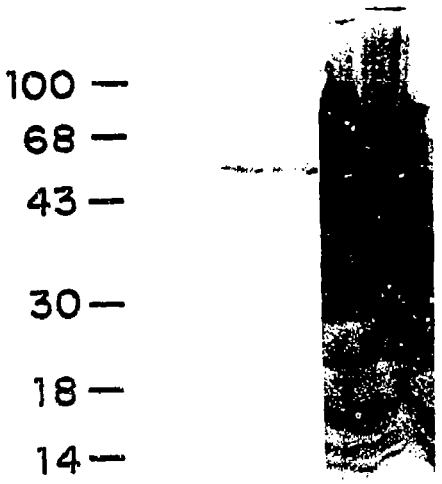

Fig. 3. Electrophoresis and immunoblot analysis of total protein from undifferentiated and differentiated neuroblastoma cells. Total protein from undifferentiated (U) and differentiated (D) N2A cells was fractionated by gel electrophoresis, transfered to nitrocellulose and tested with anti-tau monoclonal antibody, 7.51.

blastoma cells cultured in the presence of [^{35}S]methionine.

A major protein with an apparent molecular weight of 64 kDa was found. Additionally, and only for differentiated cells, a 100 kDa protein was observed. In some cases minor peptides, with a molecular weight below 45 kDa were observed for differentiated and undifferentiated cells (data not shown). Both 100 and 64 kDa proteins were heat resistant, a property similar to that of brain tau [31]. By Western blot analyses, using a whole cell protein extract, it was found that the 64 kDa protein reacts against an anti-tau antibody (7.51) which recognizes a region close to the carboxy-terminus of brain tau [37] (Fig. 3), and with a polyclonal antibody against tau, raised against a peptide included in the second tubulin-binding motif [9] of brain tau (not shown). Additionally, in the case of differentiated neuroblastoma cells, the 100 kDa protein reacted with anti-tau antibodies. However, no reaction was found for neuroblastoma tau-related proteins, with an antibody (133) that recognizes an epitope located at the amino-terminus of brain tau [37].

3.3. *Characterization of tau RNA transcribed on neuroblastoma cells*

The function of tau in microtubule stabilization probably depends on its interaction with tubulin, which itself depends on the number of tubulin-binding motifs, being greater for tau containing four motifs than for tau containing three motifs [16]. Thus, we have tested if there is a change in the population of tau containing three or four tubulin-binding motifs during neuroblastoma cell differentiation. Since we do not have an antibody which specifically recognizes the extra tubulin-binding motif we have done the test by analyzing tau RNA transcribed in undifferentiated and differentiated neuroblastoma cells.

Total RNA was prepared from undifferentiated and differentiated cells and used, together with the oligonucleotides R1 and R2 or R1 and Ct (see section 2.4. and Fig. 4), for cDNA synthesis. We have used these oligonucleotides since, as previously indicated, we found that neuroblastoma tau-related proteins contain sequences related to those of brain tau present at the tubulin-binding motif and at the carboxy-terminus, but do not contain some sequences located at the amino-terminus of brain tau. PCR amplification was done with the individual cDNA products, and the amplified cDNA from undifferentiated and differentiated cells was characterized. Three types of analyses were done that are shown in Fig. 5. Fig. 5A shows the size distribution of amplified cDNA from undifferentiated and differentiated cells synthetized in the presence of R1 and Ct oligonucleotides, while Fig. 5B shows those synthetized in the presence of R1 and R2. In both cases, two prominent cDNA bands with a difference in size of 100 base pairs were found. For undifferentiated cells, a similar

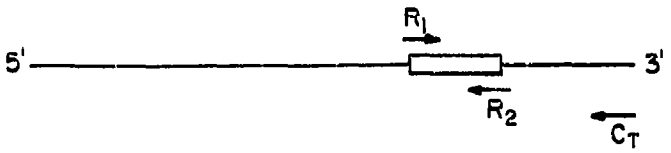

Fig. 4. Schematic map of tau cDNA. The box shows the tubulin-binding region. Arrows indicate the positions and orientation of where the synthetic oligonucleotides (used to specifically prime cDNA synthesis) bind. The sequences for the oligonucleotides R1, R2 and Ct are indicated in section 2.

proportion of each band was found, while for differentiated cells a larger proportion was observed for the slower migrating band.

The previous result is compatible with the presence of two cDNA populations; one containing three tubulin-binding regions (the faster migrating band), and the other containing an extra fourth tubulin-binding motif (the slower migrating band) [9,12–15].

A second type of analysis was the Southern blot to show that indeed the previous cDNA were tau cDNAs. Fig. 5C shows that it is indeed the case, since both bands reacted with tau cDNA.

A third type of analysis, to look for the presence of an extra tubulin-binding motif in the slower migrating band, was to test the restriction map of both cDNAs upon cleavage with *Sma*I restriction endonuclease, which should cleave the larger but not the smaller cDNA fragment according to the nucleotide sequence for cDNA tau containing three and four tubulin-binding motifs [13,14], and with *Hin*dIII, which should yield similar restriction maps. Fig. 5D shows that this appears to be the case, since a similar map was found for both fragments after *Hin*dIII incubation while differences were observed upon treatment with *Sma*I endonuclease.

4. DISCUSSION

During neuroblastoma cell differentiation a net increase in the amount of microtubule polymer has been extensively described [18,20,21]. This increase has been correlated with an increase in the phosphorylation of beta tubulin subunit and MAP_1B [18–21], but other factors, like tau factor, could be involved in the change of polymer amount. The presence of tau-related protein in neuroblastoma cells has been observed by immunofluorescence, and their characterization based on their solubility in perchloric acid, precipitation with glycerol, heat resistance and reaction with anti-tau antibodies indicate common features between these proteins and brain tau. Tau has been involved in microtubule stabilization [7] and since that stabilization may be greater for tau isoforms containing four tubulin-binding motifs that for those isoforms containing three motifs [16] we have tested whether, during neuroblastoma differentiation, there is a change in the proportion of one or the other isoform class. To do that, tau RNA transcribed in undifferentiated or differentiated neuroblastoma cells was characterized.

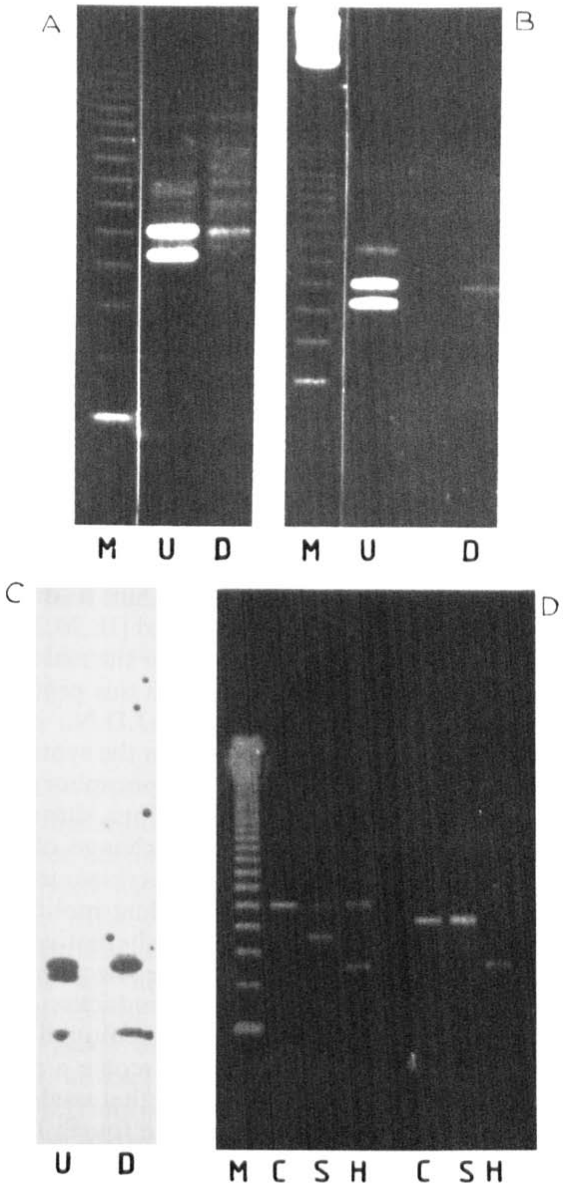

Fig. 5. Characterization of tau cDNAs. Total RNA from undifferentiated (U) and differentiated (D) cells were mixed with oligonucleotides R1 and CT (A) or with oligonucleotides R1 and R2 (B), for cDNA synthesis. PCR amplification was done with the individual cDNA products in the presence of the respective oligonucleotides. The amplified cDNAs were characterized by gel electrophoresis. M shows the DNA fragments of 123, 246, 369, 492, 615, ... base pairs used as eletrophoretic markers. (C) Southern blot analysis of the cDNAs as indicated in section 2. It shows that tau DNA binds to both cDNA fragments present in a larger amount in (A) and (B). (D) Tau cDNAs isolated in (A) and (B) were digested with restriction nucleases *Hin*dIII (H), *Sma*I (S) or undigested (C) and the resulting fragments were characterized by gel electrophoresis. On the left of the figure are shown the same markers used in (A) and (B).

Our results have shown that in undifferentiated mouse N2A neuroblastoma cells two RNA populations, coding for the tau region containing the tubulin-

binding motifs, are present. One population will be translated into a protein that contains three tubulin-binding motifs, while the other has an extra motif. A similar proportion of each population was found for RNA populations in undifferentiated cells. However, in morphologically differentiated cells, essentially only the population containing four motifs was observed. This result suggests that the 100 kDa tau-related proteins present only in differentiated cells may contain four tubulin-binding motifs, like the majority of the other tau-related proteins present in differentiated cells. A possible consequence of the increase of tau-related proteins containing four tubulin motifs will be a higher stabilization of cell microtubules that could also result in a decrease in their depolymerization, and if no changes in polymerization take place the consequence will be a net microtubule polymerization.

During differentiation of N2A neuroblastoma cells a change in the phosphorylation of two microtubule proteins, MAP_1B and tubulin, without a dramatic change in their mass, has been described [18,20,21]. For tau-related proteins no main change for the mass of the 64 kDa protein was observed although this protein becomes modified by phosphorylation (J.D.N., preliminary observations), while an increase in the synthesis of the 100 kDa protein (but not in its phosphorylation; I.C., unpublished results) was found. Thus, during neuroblastoma differentiation the main change observed for tau-related proteins was the increase in those isoforms containing four tubulin-binding motifs, after selection of a preferential way for alternative RNA splicing. A possible exploration for the selection of this alternative RNA splicing may be the induction of specific factors upon serum withdrawal in cultured neuroblastoma cells. These factors may overcome a possible steric interference between splice sites that could block the splicing of the exon containing the fourth tubulin-binding motif in a way similar to that described for the splicing block of a short c-src neuron-specific exon in non-neural cells [35]. Other cases for the selection of an alternative way of RNA splicing have been indicated for other RNAs upon neuroblastoma differentiation, such as that of β/A4 amyloid RNA [36] that is translated into a protein that has been related, like tau protein, some features in Alzheimer's disease.

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