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Depletion of casein kinase II by antisense oligonucleotide prevents neuritogenesis in neuroblastoma cells

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Casein kinase II is a multifunctional protein kinase which has been implicated in the regulation of cell growth and differentiation. This enzyme is much more abundant in neurons than in any other cell type. The treatment of neuroblastoma cells with an antisense oligodeoxyribonucleotide which specifically results in the depletion of casein kinase II catalytic subunits blocks neuritogenesis. Accordingly, this enzyme may perform an essential role during neurite growth in developing neurons. Casein kinase II depletion induced by antisense oligodeoxyribonucleotide is accompanied by a site-specific dephosphorylation of microtubule-associated protein MAP1B (also referred to as MAP5, MAP1.X or MAP1.2), which is paralleled by a release of MAP1B from microtubules. We therefore propose that phosphorylation by casein kinase II may be required for the proper MAP1B functioning in the promotion of the assembly of microtubules which constitute the cytoskeletal scaffolding of growing axon-like neurites.

Key words: antisense oligonucleotide/axonal growth/casein kinase II/microtubule-associated proteins/protein phosphorylation

Introduction

Casein kinase II (CKII) is a multifunctional serine/threonine-specific protein kinase which can phosphorylate a variety of substrates, including metabolic enzymes, cytoskeletal proteins, transcription factors and the products of several oncogenes (Edelman *et al.*, 1987; Carroll *et al.*, 1988; Krebs *et al.*, 1988; Schneider and Issinger, 1989; Pinna, 1990; Tuazon and Traugh, 1991). The enzyme has been purified from various tissues from several organisms and appears to be an oligomeric protein with the structure $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ (Edelman *et al.*, 1987; Pinna, 1990; Tuazon and Traugh, 1991). Molecular cloning and sequencing of cDNAs from different species has revealed an extremely high degree of structural conservation of CKII subunits during evolution (Sakena *et al.*, 1987; Jacoby *et al.*, 1989; Meisner *et al.*, 1989; Lozeman *et al.*, 1990; Pinna, 1990; Boldyreff *et al.*, 1991; Maridor *et al.*, 1991; Tuazon and Traugh, 1991). The molecular weights of CKII subunits predicted from the human cDNAs are 45, 160 (α), 41, 450 (α') and 24, 925 (β) (Lozeman *et al.*, 1990). The α and α' subunits are catalytic, whereas the β subunit is thought to stabilize the holoenzyme and favour the recognition of specific substrates (Grankowski *et al.*, 1991; Lin *et al.*, 1991).

The biological roles and the regulatory mechanisms of CKII in mammalian cells remain to be fully understood. A great deal of research has recently been devoted to study CKII in proliferating non-neuronal cells. CKII appears to play a major role in the mitogenic signalling elicited by several growth factors (Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Krebs *et al.*, 1988; Ackerman and Osheroff, 1989; Carroll and Marshak, 1989; Ackerman *et al.*, 1990; Litchfield *et al.*, 1991; Pepperkok *et al.*, 1991). Phosphorylation of nuclear proteins by CKII may also be involved in the control of nucleocytoplasmic protein transport (Rihs *et al.*, 1991) and in the regulation of transcription (Carroll *et al.*, 1988; Lüscher *et al.*, 1989; Meek *et al.*, 1990; Meisner and Czech, 1991; Lin *et al.*, 1992). Additionally, the phosphorylation of cytoskeletal proteins by CKII might contribute to the structural rearrangements underlying mitosis (Serrano *et al.*, 1989; Mulner-Lorillon *et al.*, 1988, 1990; Yu *et al.*, 1991; Krek *et al.*, 1992; Díaz-Nido and Avila, 1992).

However, CKII is much more abundant in brain than in any other tissue (Girault *et al.*, 1990; Krek *et al.*, 1992). Furthermore, immunohistochemical studies show that CKII is more concentrated in neurons than in glial cells (Iimoto *et al.*, 1989; Girault *et al.*, 1990), suggesting a potentially important role for this enzyme in specific neuronal functions. In particular, one of the proposed roles for casein kinase II in developing neurons has been the promotion of neuritogenesis through the phosphorylation of several cytoskeletal proteins including microtubule-associated protein MAP1B (Díaz-Nido *et al.*, 1988, 1990a, 1991, 1992; Serrano *et al.*, 1989) and the neuronal-specific βIII tubulin (Serrano *et al.*, 1987; Díaz-Nido *et al.*, 1990a,b).

In this report we have used antisense oligonucleotide treatment to study the effect of a transient CKII depletion on the capacity of neuroblastoma cells to generate neurites. Neuroblastoma cell cultures have been considered a particularly suitable model system to analyse molecular mechanisms implicated in neuritogenesis (Seeds *et al.*, 1970; Yamada *et al.*, 1970, 1971; Prasad, 1975; Gard and Kirschner, 1985; Eddé *et al.*, 1987; Díaz-Nido *et al.*, 1988, 1991, 1992). Antisense oligonucleotides are a powerful tool to inhibit the function of specific mRNAs, possibly due to hybrid formation and subsequent RNase activation, thus hindering *de novo* protein synthesis and causing depletion of the respective proteins in cultured cells (Zamecnik and Stephenson, 1978; Akhtar and Juliano, 1992; Akhtar *et al.*, 1992; Agrawal, 1992). For instance, antisense oligonucleotides have been used to study the effect of depletion in tubulin (Teichman-Weinberg *et al.*, 1988), MAP1B (Brugg *et al.*, 1993), tau (Cáceres and Kosik, 1990; Cáceres *et al.*, 1991) or peripherin (Troy *et al.*, 1992) on neuritogenesis. Likewise, an important role for CKII in the mitogenic signalling elicited by EGF has been demonstrated through the use of antisense oligonucleotides (Pepperkok *et al.*, 1991).

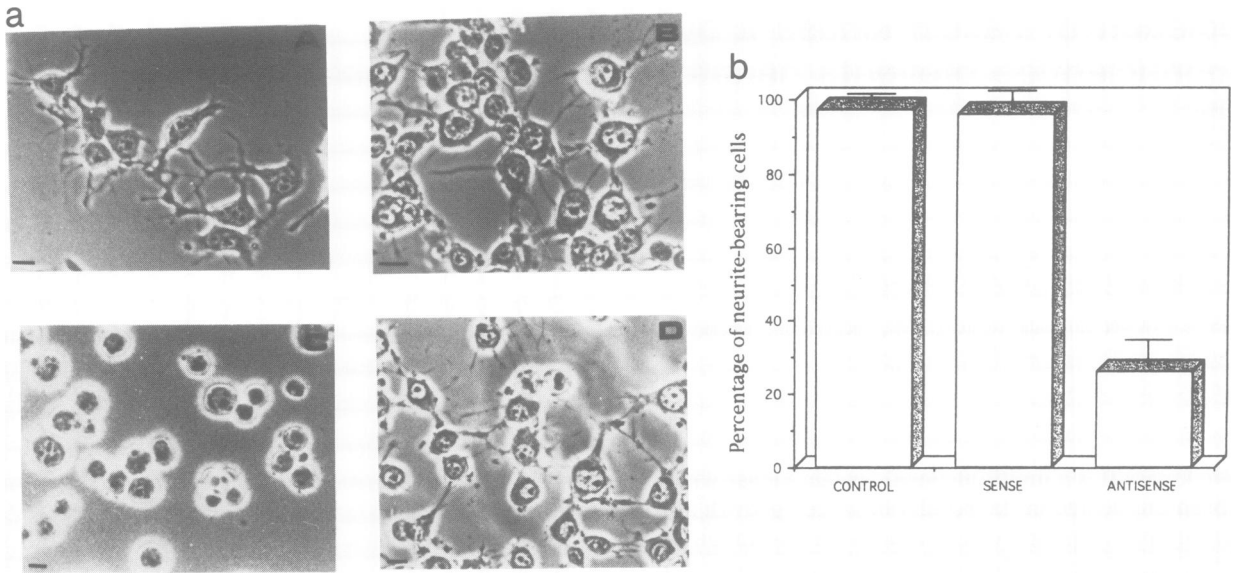

Fig. 1. Effect of casein kinase II antisense oligonucleotide on neurite outgrowth in neuroblastoma cells. (**a**) Phase contrast microphotographs of neuroblastoma N2A cells maintained in serum-free medium for 8 h without any addition (A), supplemented with a sense oligonucleotide corresponding to a conserved sequence in the cDNAs coding for catalytic subunits (α + α') of CKII (B), supplemented with the corresponding antisense oligonucleotide (C) or supplemented with the mixture of the previously complexed sense and antisense oligonucleotides (D). Oligonucleotides were added to culture medium at a concentration of 0.3 mg/ml 2 h prior to serum deprivation. They were added again at the time of serum withdrawal and fresh medium containing new oligonucleotides was given to the cells every 1.5 h. (**b**) Quantification of the effect of CKII antisense oligonucleotide on neurite outgrowth in neuroblastoma N2A cells. To quantify neurite outgrowth, 50 cells from each of four randomly chosen fields in each of five wells were observed after methanol fixation and phase-contrast microphotography. Data are mean ± SD (bars) values.

Results

Inhibition of neurite outgrowth by casein kinase II (α/α' subunit) antisense oligonucleotide

The addition of a CKII antisense oligodeoxyribonucleotide to the culture medium of mouse neuroblastoma N2A cells produces a dramatic inhibition of neurite outgrowth. Figure 1 shows the morphology of N2A cells extending neurites in serum-free medium. In the presence of CKII (α/α' subunit) antisense oligonucleotide, neurite extension is prevented and cells become rounded (Figure 1), whereas neuritogenesis is observed either in the presence of the complementary sense oligonucleotide or in the presence of both (sense and antisense) oligonucleotides mixed before adding them to the culture medium.

The expected depletion of CKII upon antisense oligonucleotide treatment was investigated by assaying the intracellular protein level of CKII α/α' subunits by immunofluorescence and immunoblotting. Figure 2 shows that there is a dramatic decrease in immunoreactivity for an antibody which recognizes CKII α and α' subunits, in cells treated with the antisense oligonucleotide, but not in untreated cells or in cells treated with the sense oligonucleotide. Moreover, as short oligonucleotides are internalized into cells via receptor-mediated endocytosis (Akhtar and Juliano, 1992; Akhtar *et al.*, 1992), the effect of antisense addition to the culture medium of N2A cells was tested in the presence of trypsin, a protease that cleaves cell surface receptors thus abolishing endocytosis. Figure 2 indicates that there is no decrease in CKII protein induced by antisense oligonucleotide when trypsin is present in the culture medium.

The effect of antisense oligonucleotide appears to be on α and α' catalytic subunits, since a lower decrease in immunoreactivity was observed with an antibody to the whole CKII holoenzyme (Figure 2 right). This suggests that no decrease in β subunit takes place after incubation with antisense oligonucleotide to CKII (α/α'). The decrease in the amount of α/α' subunit in neuroblastoma cells treated with antisense oligonucleotide was confirmed by immunoblotting analysis (Figure 2).

The inhibition of neurite outgrowth by antisense oligonucleotides is transient

The time course of neurite outgrowth from N2A cells maintained in serum-free medium either in the absence or in the presence of the CKII antisense oligonucleotide shows that the inhibition of neuritogenesis induced by the antisense oligonucleotide is transient (Figure 3). Resumption of neurite growth after 2 h in the presence of the antisense oligonucleotide is accompanied by the reappearance of immunoreactivity for CKII (α/α') and is prevented by new addition of the oligonucleotide (not shown). This indicates that the transient effect on both CKII depletion and neuritogenesis blockage is due to the lack of stability of short oligodeoxyribonucleotides, which may be rapidly degraded by intracellular and extracellular nucleases as previously described (Akhtar *et al.*, 1992). More importantly, this also demonstrates that the inhibition of neuritogenesis after treatment with the CKII antisense oligonucleotide is reversible and therefore not the result of cell damage due to toxic effects of oligonucleotides. Complete reversibility of neurite outgrowth and no decrease in cell viability (as determined by trypan blue exclusion) is also observed when N2A cells are maintained for up to 8 h in the presence of antisense oligonucleotide (with new additions every 1.5 h period).

Depletion of casein kinase II is accompanied by site-specific dephosphorylation of microtubule-associated protein MAP1B

It has been indicated that CKII may be involved in the phosphorylation of MAP1B which occurs during neurite

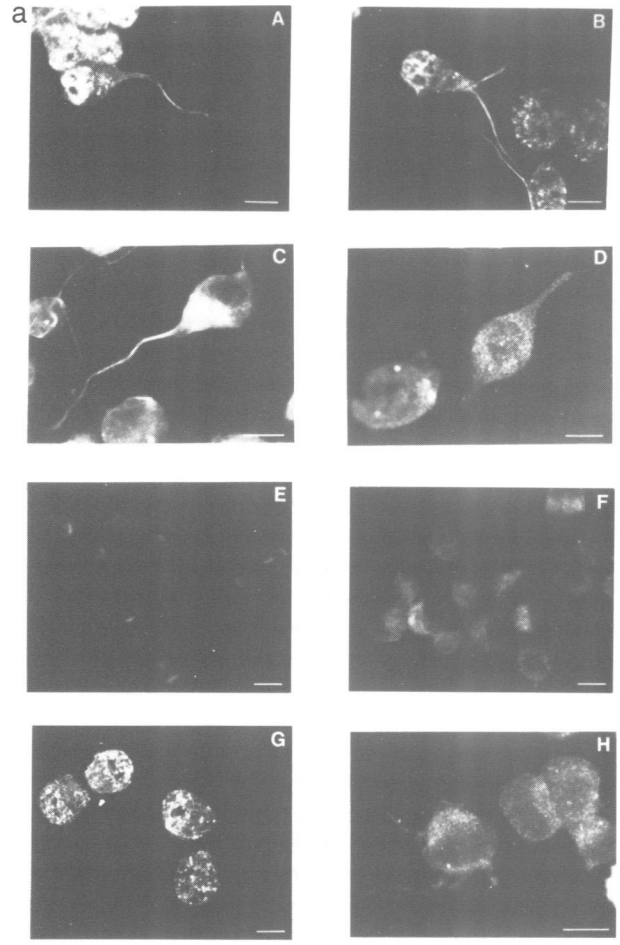

Fig. 2. Depletion of casein kinase II catalytic subunits in neuroblastoma cells upon antisense oligonucleotide treatment. (**a**) Immunofluorescence staining patterns of neuroblastoma N2A cells maintained in serum-free medium without any addition (**A** and **B**), supplemented with the sense oligonucleotide to CKII (**C** and **D**), supplemented with the antisense oligonucleotide to CKII (**E** and **F**) or supplemented with the sense oligonucleotide to CKII in the presence of trypsin which inhibits receptor-mediated endocytotic oligonucleotide uptake (**G** and **H**) and incubated either with an antibody to CKII catalytic subunit (**A**, **C**, **E** and **G**) or an antibody to CKII holoenzyme which also reacts with the regulatory β subunit (**B**, **D**, **F** and **H**). More than 80% of cells exhibited the patterns shown in the micrographs after examination of 100 cells from randomly chosen fields for each culture condition. Bar represents 25 μm. (**b**) Immunoblotting analyses showing the reaction of the antibody to CKII catalytic subunit (45 kDa) with aliquots containing the same amount of protein of extracts obtained from cells maintained in serum-free medium without any addition (C), supplemented with the sense oligonucleotide to CKII (S) or supplemented with the antisense oligonucleotide to CKII (A).

extension in neuroblastoma cells (Díaz-Nido *et al.*, 1988). To test for the effect of CKII depletion on the phosphorylation of MAP1B, a monoclonal antibody (125) reacting with a phosphorylated epitope on MAP1B was used.

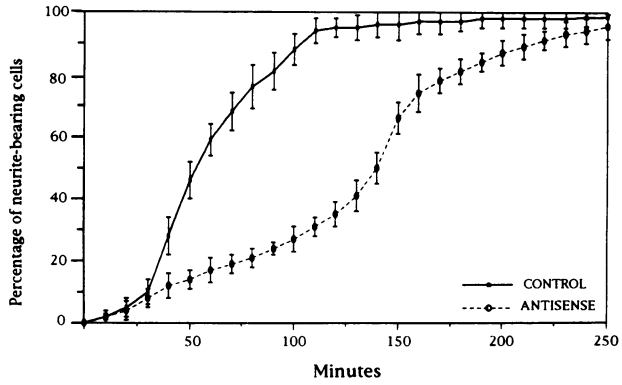

Fig. 3. Inhibition of neuritogenesis by casein kinase II antisense oligonucleotide is transient. Temporal course of neurite outgrowth in serum-free medium either in the absence (CONTROL) or in the presence of the antisense oligonucleotide to CKII (ANTISENSE). Resumption of neurite growth after 2 h in the presence of the antisense oligonucleotide is accompanied by reappearance of immunoreactivity for antibodies to CKII (not shown). Quantification of neurite outgrowth is performed as in the legend to Figure 1b.

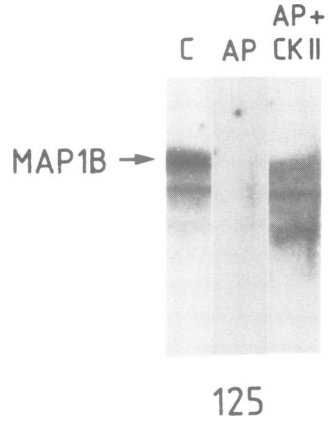

Fig. 4. Phosphorylation of the site constituting the 125 epitope by purified casein kinase II *in vitro*. Immunoblotting analyses showing the reaction of antibody 125 with aliquots containing the same amount of protein (30 μg) from MAPs prepared from 5 day old rat brain and devoid of endogenous protein kinases (C), subsequently treated with alkaline phosphatase (AP), and phosphorylated *in vitro* by purified casein kinase II after alkaline phosphatase treatment (AP + CKII) as described in Materials and methods. Arrow marks the position of MAP1B. Note the reaction of antibody 125 with a 260 kDa polypeptide derived from MAP1B proteolysis which has been previously reported (Riederer *et al.*, 1986; Díaz-Nido and Avila, 1989). It is observed that MAP1B proteolysis is enhanced by alkaline phosphatase treatment, possibly due to the presence of minor traces of proteases in the phosphatase preparation.

The epitope for antibody 125 on rat brain MAP1B is lost upon exhaustive treatment with alkaline phosphatase (Figure 4, lane AP), and reappears after the phosphorylation of phosphatase-treated MAP1B with purified casein kinase II *in vitro* (Figure 4, lane AP+CKII). Accordingly, antibody 125 recognizes a site phosphorylatable by CKII.

Figure 5 shows that the incubation of N2A cells with CKII antisense oligonucleotide results in a decrease in the immunoreactivity of MAP1B for antibody 125 without affecting its immunoreactivity for antibody AA6 (which recognizes a phosphorylation-independent epitope on MAP1B) as determined by immunofluorescence microscopy and immunoblotting assay. Additionally, no effect is found when the sense oligonucleotide is tested. The loss of immunoreactivity for antibody 125 in cells depleted of CKII suggests that phosphorylation at the site constituting the 125

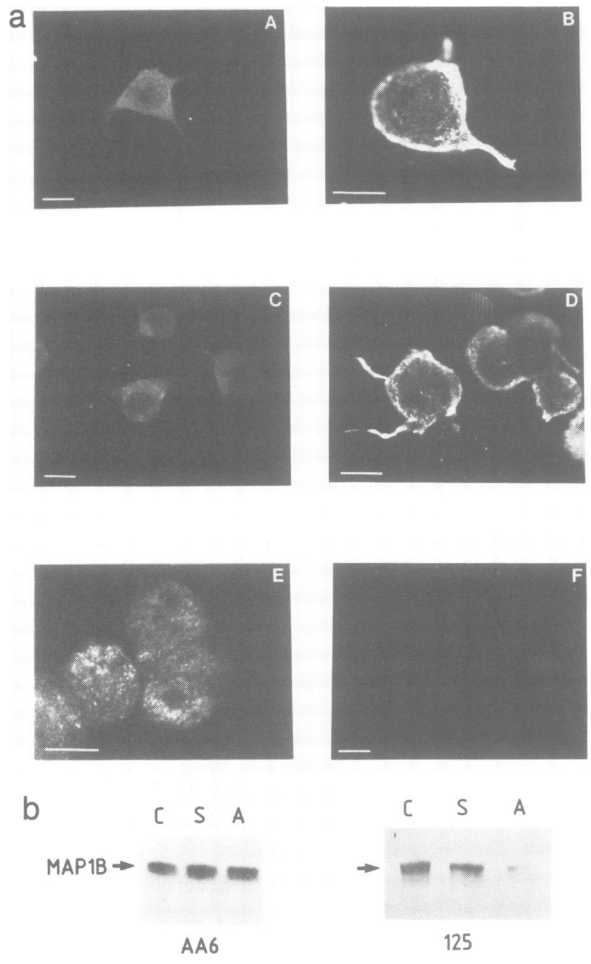

Fig. 5. Loss of immunoreactivity for antibody 125 recognizing phosphorylated MAP1B in neuroblastoma cells treated with casein kinase II antisense oligonucleotide. (**a**) Immunofluorescent staining patterns of neuroblastoma N2A cells maintained in serum-free medium without any addition (**A** and **B**), supplemented with the sense oligonucleotide to CKII (**C** and **D**) or supplemented with the antisense oligonucleotide to CKII (**E** and **F**) and incubated either with antibody AA6 (A, C and E) or antibody 125 to MAP1B (B, D and F). More than 80% of cells exhibited the pattern shown in the micrographs after examination of 100 cells from randomly chosen fields for each culture condition. Bar represents 25 μm. (**b**) Immunoblotting analyses showing the reactions of antibodies AA6 (left) and 125 (right) with aliquots containing the same amount of protein of extracts from cells maintained in serum-free medium without any addition (C), supplemented with the sense oligonucleotide to CKII (S) or supplemented with the antisense oligonucleotide to CKII (A). Arrows mark the position of MAP1B.

epitope on MAP1B may also be catalysed by CKII *in vivo*. This lack of immunoreactivity of MAP1B for antibody 125 also indicates the occurrence of dephosphorylation at the site recognized by antibody 125, as a small fraction of MAP1B is phosphorylated at this site even in undifferentiated cells (not shown) prior to the treatment with antisense oligonucleotides. These results are consistent with the suggested importance of protein phosphatases in controlling the constitutive phosphorylation of a number of CKII substrates (Pinna, 1990).

Interestingly, CKII depletion, which almost abolishes immunoreactivity for antibody 125, does not reduce immunoreactivity for antibody SMI-31, which reacts with another phosphorylated epitope on MAP1B (Figure 6). Although the epitope recognized by SMI-31 is shared by MAP1B and neurofilaments (Fischer and Romano-Clarke, 1990), the immunoreactivity observed in our neuroblastoma cells mainly corresponds to MAP1B, as indicated by immunoblotting (Figure 6). The phosphorylation of the NF-H neurofilament protein subunit is only observed in N2A cells within long neurites after 3–4 days of serum deprivation, which is compatible with the results obtained for NB2a/d1 cells by Shea *et al.* (1989).

Treatment of N2A cells with the specific protein kinase inhibitor DRB abolishes immunoreactivity for both antibodies 125 and SMI-31 and also results in inhibition of neuritogenesis (Figure 6). DRB was originally described as a highly specific CKII inhibitor (Zandomeni *et al.*, 1986), but it has recently been reported that it also inhibits at least some members of the closely related group of proline-directed protein kinases (PDPKs) (Stevens and Maupin, 1989). This suggests that phosphorylation at the site constituting the SMI-31 epitope on MAP1B is independent of CKII and may be catalysed by a DRB-sensitive PDPK. This is compatible with the fact that the epitope for SMI-31 contains phosphorylated serine followed by proline residues within consensus targets for PDPKs (Lee *et al.*, 1988). These data demonstrate that CKII depletion results in the dephosphorylation of specific sites without affecting other phosphorylation sites on the MAP1B molecule.

Effect of site-specific dephosphorylation of MAP1B on its association with microtubules in situ

To test for the functional consequences of the altered phosphorylation state of MAP1B in neuroblastoma cells depleted of CKII, the association of this protein with microtubules has been examined. To do it, cells were permeabilized with a non-ionic detergent, Triton X-100, which extracts soluble cytoplasmic proteins. Then, the assembled microtubules were depolymerized and extracted with a cold calcium-containing buffer as previously described (Black *et al.*, 1984; Díaz-Nido *et al.*, 1990b). Figure 7a shows that the amount of MAP1B bound to microtubules is substantially lower in cells depleted of CKII than in control cells, as visualized by immunofluorescence microscopy with the antibody AA6. Immunoblotting assays with this antibody demonstrates the occurrence of an increased soluble pool of MAP1B together with a decreased pool of microtubule-bound MAP1B in cells depleted of CKII (Figure 7b). These results suggest that phosphorylation of MAP1B by CKII is probably required for an efficient association with microtubules. As this association seems to be essential for the consolidation of neurite outgrowth (Matus, 1991; Brugg *et al.*, 1993), it may be hypothesized that the lack of phosphorylation of MAP1B by CKII may be responsible for the observed block in neuritogenesis in cells treated with the CKII antisense oligonucleotide.

Curiously, a substantial amount of MAP1B is found associated with the remaining cytoskeletal fraction after microtubule extraction (Figure 7b). This pool of MAP1B is not decreased but appears slightly increased in cells depleted of CKII (Figure 7b). Previous data have shown the presence of MAP1B in particulate fractions obtained from rat brain homogenates (Riederer *et al.*, 1991) and in the cytoskeletal fractions obtained from growth cone preparations (Mansfield *et al.*, 1992). This pool of MAP1B may correspond to MAP1B bound to actin microfilaments and other cytoskeletal elements. A co-localization of MAP1B with actin in certain non-neuronal cells has been described

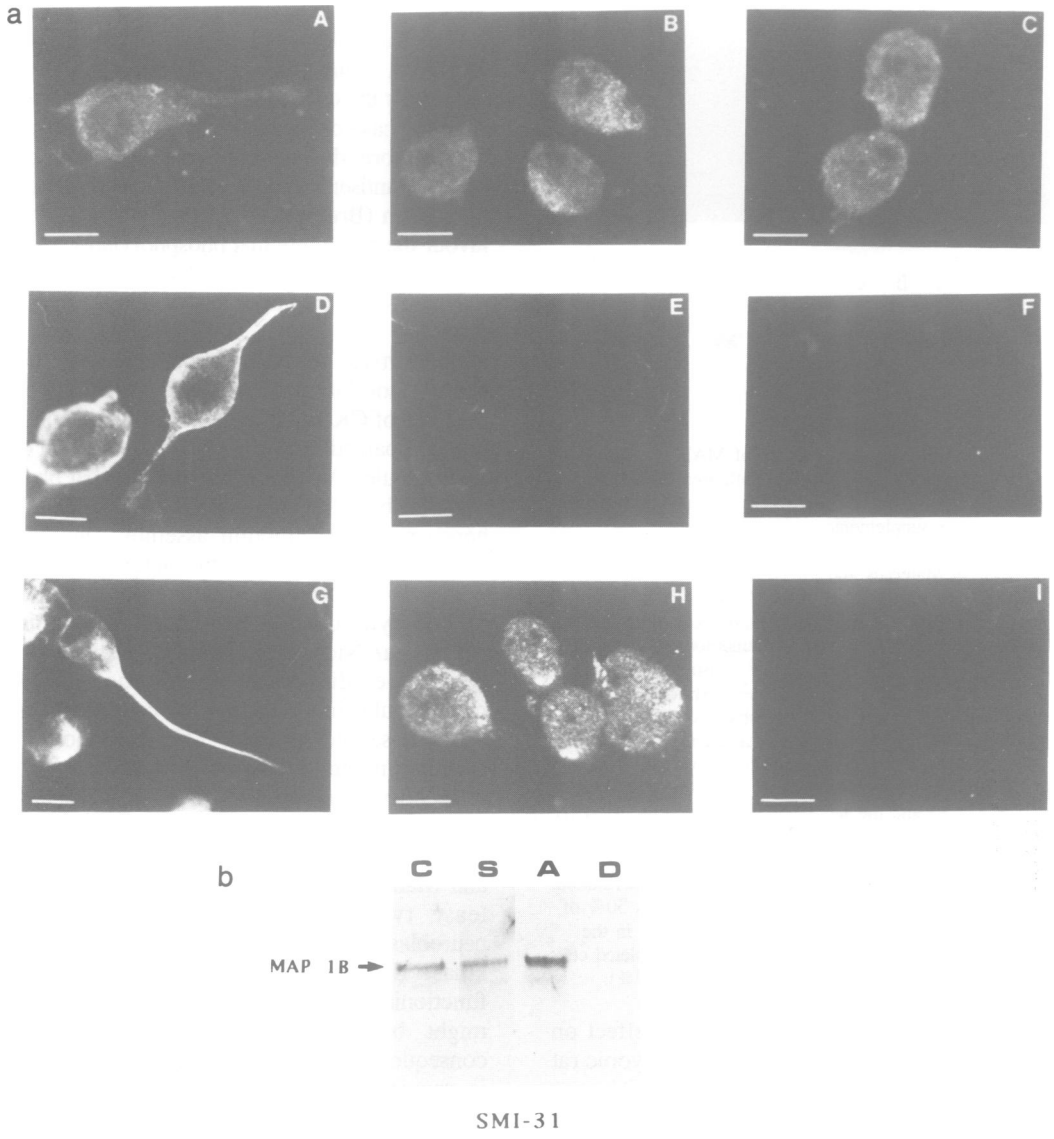

Fig. 6. Loss of immunoreactivity for antibodies 125 and SMI-31 in neuroblastoma cells treated with the protein kinase inhibitor DRB. (**a**) Immunofluorescent staining patterns of neuroblastoma N2A cells maintained in serum-free medium (**A**, **D** and **G**), supplemented with the antisense oligonucleotide to CKII (**B**, **E** and **H**) or supplemented with DRB (**C**, **F** and **I**) and incubated with antibodies AA6 (A, B and C), 125 (D, E and F) or SMI-31 (G, H and I). More than 80% of cells exhibited the patterns shown in the micrographs after examination of 100 cells from randomly chosen fields. (**b**) Immunoblotting analyses showing the reaction of antibody SMI-31 with MAP1B from extracts containing the same amount of protein and obtained from cells maintained in serum-free medium (C), supplemented with the sense oligonucleotide to CKII (S), supplemented with the antisense oligonucleotide to CKII (A) or supplemented with DRB (D). Arrow marks the position of MAP1B.

(Asai *et al.*, 1985). Furthermore, MAP1B is fairly abundant within actin-rich filopodia completely devoid of microtubules in taxol-treated axonal growth cones (Mansfield *et al.*, 1992). However, this pool of MAP1B which is possibly associated with actin microfilaments cannot be visualized by immunofluorescence using methanol fixation, as used here, since this procedure disrupts microfilaments (Vielkind and Swierenga, 1989). Additionally, a large fraction of MAP1B is associated with the microtubule-organizing centres which partitions with the cytoskeletal fraction (Díaz-Nido and Avila, 1989).

Discussion

The inhibition of neuritogenesis observed after casein kinase II (CKII) depletion induced by treatment of neuroblastoma N2A cells with a specific antisense oligodeoxyribonucleotide demonstrates an essential role for CKII in controlling neurite growth. In fact, a translocation of the enzyme from the nucleus, where it is largely confined in proliferating cells (Serrano *et al.*, 1989; Filhol *et al.*, 1990; Yu *et al.*, 1991; Díaz-Nido *et al.*, 1992; Krek *et al.*, 1992), to the cytoplasm has been observed in differentiating neuroblastoma cells (Serrano *et al.*, 1989; Díaz-Nido *et al.*, 1991, 1992). We suggest that certain phosphorylatable cytoskeletal proteins including microtubule-associated protein MAP1B may be the targets for cytosolic CKII implicated in neuritogenesis.

The effect of CKII depletion on neuritogenesis cannot be explained as arising from a general deficit in the rate of protein synthesis, as rapid neurite outgrowth in neuroblastoma cells is largely insensitive to inhibitors of protein synthesis or RNA transcription (Seeds *et al.*, 1970).

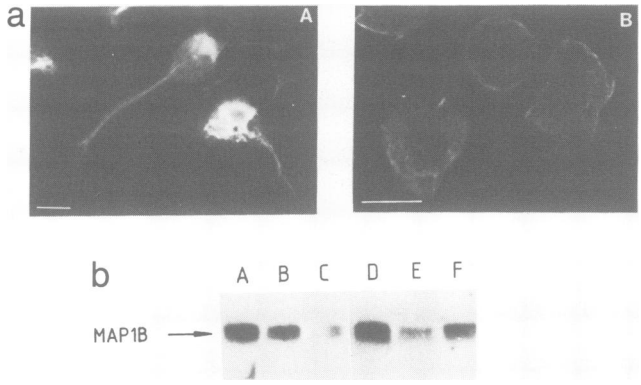

Fig. 7. Effect of site-specific dephosphorylation of MAP1B on its association with microtubules *in situ*. (**a**) Immunofluorescent staining pattern of microtubules from neuroblastoma N2A cells maintained in serum-free medium (**A**), or supplemented with the antisense oligonucleotide to CKII (**B**), after their extraction with a non-ionic detergent, as described in Materials and methods, prior to their incubation with the monoclonal antibody AA6. About 80% of cells exhibited the patterns shown in the micrographs after examination of 100 cells from randomly chosen fields. (**b**) Immunoblotting analyses showing the presence of MAP1B in the cold/Ca^{2+}-insoluble cytoskeletal protein fractions (**A** and **D**), cold/Ca^{2+}-extracted microtubule protein fractions (**B** and **E**) and soluble protein fractions (**C** and **F**) prepared from N2A cells either maintained in serum-free medium (A, B and C) or treated with the antisense oligonucleotide to CKII (D, E and F). Note the decreased pool of microtubule-associated MAP1B (compare B and E) and the increased pool of soluble MAP1B (compare C and F) in CKII-depleted cells. Densitometric scanning of several blots from different experiments indicate that 45% of MAP1B is in the cold/Ca^{2+}-insoluble cytoskeletal pool, 35% in the microtubule pool and 15% in the soluble pool from control cells, whereas 50% of MAP1B is in the cold/Ca^{2+}-insoluble cytoskeletal pool, 18% in the microtubule pool and 35% in the soluble pool from CKII-depleted cells (standard deviations of these mean values are in all cases <4%).

Likewise, inhibition of protein synthesis has no effect on the initiation of axonal growth from cultured embryonic rat sympathetic neurons (Lein and Higgins, 1991), which may be considered as the normal counterparts to neuroblastoma cells.

Neurite outgrowth clearly depends on the increased assembly of microtubules which constitute the cytoskeletal scaffolding of developing neurites (Seeds *et al.*, 1970; Yamada *et al.*, 1970, 1971). As this increased assembly of microtubules is not paralleled by any change in the amount of tubulin present in neuroblastoma cells (Olmsted, 1981), a major emphasis has been placed on post-translational modifications of pre-existing proteins (Gard and Kirschner, 1985; Eddé *et al.*, 1987; Serrano *et al.*, 1987; Díaz-Nido *et al.*, 1988, 1990a,b, 1991, 1992). Of particular interest, in this regard, is the phosphorylation of microtubule-associated proteins (MAPs), as it might modulate the ability of these proteins to promote microtubule assembly.

Thus, the enhanced phosphorylation of microtubule-associated protein MAP1B (also referred to as MAP5, MAP1.X or MAP1.2) has been shown to take place during neurite outgrowth (Gard and Kirschner, 1985; Aletta *et al.*, 1988; Díaz-Nido *et al.*, 1988). MAP1B is a good candidate to be the protein responsible for promoting microtubule assembly within growing axon-like neurites (Matus, 1991; Brugg *et al.*, 1993). MAP1B seems to be the first MAP expressed in developing neurons both at the central and the peripheral nervous systems (Tucker and Matus, 1988; Tucker *et al.*, 1988, 1989) and is actually the major MAI in neuronal cell lines such as PC12 (Brugg and Matus, 1988 and N2A (Díaz-Nido *et al.*, 1991). Indeed, MAP1B i practically the only MAP present within certain axons *in situ* as in the case of the olfactory nerve (Viereck *et al.*, 1989) Furthermore, depletion of MAP1B in PC12 cells treated wit MAP1B antisense oligonucleotides completely blocks neurit outgrowth (Brugg *et al.*, 1993). In view of these data, w favour the possibility that phosphorylation of MAP1B, whic is an excellent substrate for CKII (Díaz-Nido *et al.*, 1988) may be the critical target for CKII in the regulation of neurit growth.

Supportive of this hypothesis is the site-specifi dephosphorylation of MAP1B which is observed in cell depleted of CKII. More importantly, this dephosphorylatio is accompanied by a diminished association of MAP1B wit microtubules. We therefore propose that phosphorylation c MAP1B by CKII may favour MAP1B binding to tubulin hence promoting tubulin assembly into the microtubule which are necessary for the outgrowth of neurites.

Previous studies have indicated that MAP1 phosphorylated with CKII can efficiently bind to tubulin *i vitro* (Díaz-Nido *et al.*, 1988). The results presented her about the diminished association of MAP1B wit microtubules in CKII-depleted cells are consistent with th effect observed *in vitro*. This phosphorylation-dependen tubulin binding may not be unique to a single MAP, as th phosphorylation of MAP2 at a particular set of site stimulates MAP2 binding to tubulin whereas phosphorylatio at another set of sites blocks MAP2 binding to tubulin (Brug and Matus, 1991). Our data also suggest that there are a least two modes of MAP1B phosphorylation i neuroblastoma cells. One of them may be catalysed by casei kinase II and might be required for the proper MAP1 functioning in promoting microtubule assembly. The othe might be catalysed by a PDPK and its functiona consequences have not been explored yet. Further researc is required to clarify the role of these two modes c phosphorylation on the association of MAP1B wit microtubules and other cytoskeletal elements.

In summary, we suggest a hypothetical model accordin to which the promotion of the assembly of tubulin int microtubules by CKII-phosphorylated MAP1B ma constitute an early stage in the consolidation of neurit outgrowth. This stage is followed by neurite elongatio which in some cases requires protein synthesis (Lein an Higgins, 1991) and might depend on the stabilization c neurite microtubules by other MAPs, including a hig molecular weight tau protein which is induced during N2 cell differentiation (Montejo *et al.*, 1992). Previous studie with antisense oligonucleotides in a variety of cultured cel of neuronal origin support this model, as the initial phas of neurite outgrowth is not observed in cells depleted of eithe tubulin (Teichman-Weinberg *et al.*, 1988) or MAP1 (Brugg *et al.*, 1993), and the subsequent phase of neurit elongation is not observed in cells depleted of tau protei (Caceres and Kosik, 1990; Caceres *et al.*, 1991).

Finally, our results showing the implication of CKII i neuritogenesis may help to understand the putative ro played by this enzyme in certain neurodegenerative disease including Alzheimer's disease. CKII is altered in cortic and hippocampal neurons from Alzheimer's disease patie brains (Iimoto *et al.*, 1990; Aksenova *et al.*, 1991; Maslia

et al., 1992; Baum *et al.*, 1992) and this might be connected with the massive and aberrant neurite regeneration which is thought to occur in Alzheimer's disease (Ihara, 1988).

Materials and methods

Oligonucleotides

CKII α/α' subunit antisense or sense oligodeoxyribonucleotides with the sequences: antisense (5′ to 3′): GTA ATC ATC TTG ATT ACC CCA or sense (5′ to 3′): TGG GGT AAT CAA GAT GAT TAC were purchased from Isogen Bioscience (Netherlands). The antisense oligonucleotide is complementary to a conserved sequence in CKII catalytic subunits (α and α') which is not found in any other protein kinase and is located in a region immediately upstream of the sequence coding for a series of three glycines characteristic of the ATP binding site (Meisner *et al.*, 1989; Lozeman *et al.*, 1990). These oligonucleotides do not match any other nucleotide sequences of known mammalian genes.

Antibodies

A rabbit polyclonal antibody to CKII holoenzyme (Dahmus, 1981) was the generous gift of Dr M.Dahmus (University of California at Davis, USA). Another rabbit polyclonal antibody to CKII ($\alpha + \alpha'$ subunits) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Antibodies to MAP1B used in this study include the mouse monoclonal antibody (IgG) AA6 (Riederer *et al.*, 1986) which is purchased from SIGMA (St Louis, MO, USA), the mouse monoclonal (IgM) antibody 125 which reacts with a phosphorylated epitope on MAP1B (L.Ulloa, J.Avila and J.Díaz-Nido, 1993), which was obtained in our laboratory, and the mouse monoclonal (IgG) antibody SMI-31 which recognizes a phosphorylated epitope shared by MAP1B and neurofilament proteins (Sternberger and Sternberger, 1983; Lee *et al.*, 1988; Fischer and Romano-Clarke, 1990), and was purchased from Sternberger–Meyer Immunochemicals (Jarretsville, MD, USA).

Cell culture

Mouse neuroblastoma (N2A) cells were grown on plastic or glass coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 0.01 mg/ml sodium pyruvate and 50 μg/ml of gentamicin at 37°C. The cells were induced to differentiate by serum deprivation (Díaz-Nido *et al.*, 1988).

Oligonucleotides were added to the culture medium at a concentration of 0.3 mg/ml and the oligonucleotide-containing medium was replaced every 1.5 h for a period of up to 6–8 h. The specific protein kinase inhibitor DRB (5, 6-dichloro-1-β-D-ribofuranosyl benzimidazole) was added to the culture medium at a concentration of 100 μM for 6–8 h.

Immunofluorescence microscopy

N2A cells grown directly on glass coverslips and induced to differentiate were fixed for 10 min in methanol at −20°C. The cells were then washed with 20 mM sodium phosphate pH 7.4, 140 mM NaCl (phosphate-buffered saline, PBS) and incubated for 1 h at room temperature with primary antibodies. The coverslips were then washed in PBS and incubated for 30 min at room temperature with a fluorescein-conjugated goat anti-rabbit or anti-mouse immunoglobulins (TAGO, Burlingame, CA), washed again with PBS and mounted with glycerol/PBS (9:1 v/v) to be examined using a Nikon epifluorescence microscope.

Protein preparation and characterization

N2A cells were washed with PBS and harvested after scraping into PBS. The cells were then collected by low-speed centrifugation and homogenized in 20 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 2 mM EGTA, 0.1% (P/V) Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin. Protein concentrations were determined using the BCA method (Smith *et al.*, 1985).

Aliquots of cell extracts were analysed by SDS–PAGE according to the procedure of Laemmli (1970) and subsequently transferred to nitrocellulose paper as described by Towbin *et al.* (1979). Transferred proteins were stained with 0.3% Ponceau-S in 3% trichloroacetic acid (TCA) for 5 min. After blocking the membranes with 2% bovine serum albumin in PBS containing 0.05% Tween-20 overnight at 4°C, the blots were incubated with primary antibodies for 2 h at room temperature. Detection of bound antibodies was performed by incubation with secondary antibodies either conjugated with alkaline phosphatase or radioiodinated.

Extraction of soluble, microtubule-bound and cytoskeletal MAP1B

Separation of soluble from microtubule-bound and cytoskeletal MAP1B was achieved by an *in situ* extraction procedure based on the method of Black *et al.* (1984). Briefly, N2A cells attached to culture dishes or coverslips were rinsed with a buffer containing 0.1 M PIPES, pH 6.9, 2 M glycerol, 1 mM $MgCl_2$, 2 mM EGTA and 10 mM NaF at room temperature, and subsequently extracted by adding a lysis buffer which consisted of 0.1 M PIPES, pH 6.9, 2 M glycerol, 1 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.3% (w/v) Triton X-100 for 10 min at room temperature. The soluble protein fraction obtained after incubation in this lysis buffer includes unassembled microtubule-associated proteins. Assembled microtubule protein partitions with the nonionic detergent-insoluble cytoskeletal fraction and was extracted by scraping the cell ghosts into a buffer containing 100 mM Tris–HCl pH 7.5, 6 mM $CaCl_2$, 0.4% (w/v) Triton-X-100, 10 mM NaF, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. After incubating for 30 min at 0°C, the extracted protein was isolated in the supernatant upon centrifugation for 15 min at 100 000 *g* at 2°C. The cold/Ca^{2+}-insoluble cytoskeletal fraction was resuspended in 20 mM Tris–HCl, pH 7.5, 5 mM EGTA, 5 mM EDTA, 10 mM DTT and 2% (w/v) SDS. The characterization of the cold/Ca^{2+}-extracted protein fraction as microtubule protein has been previously described (Black *et al.*, 1984; Díaz-Nido *et al.*, 1990b). Aliquots of the soluble, the cold/Ca^{2+}-extracted microtubule and the cold/Ca^{2+}-insoluble cytoskeletal protein fractions were analysed by gel electrophoresis and immunoblotting as described above. Alternatively, cells on coverslips were extracted with the lysis buffer and processed for immunofluorescence microscopy as previously indicated.

In vitro phosphorylation assay

A preparation of MAPs enriched in MAP1B and devoid of endogenous protein kinases was obtained from 5 day old rat brains as described by Díaz-Nido *et al.* (1988). MAPs were incubated with alkaline phosphatase-coated acrylic beads (Sigma, Cat. No. PO927) in 100 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 50 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml leupeptin for 4 h at room temperature. The sample was then centrifuged and the resulting supernatant boiled to inactivate minor traces of alkaline phosphatase. Dephosphorylated MAPs were phosphorylated *in vitro* with added casein kinase II, 5 mM ATP and 2 μg/ml poly-L-lysine for 5 h at 37°C. Casein kinase II was purified from rat brain according to the method of Alcázar *et al.* (1988).

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