Addition of protease inhibitors to culture medium of neuroblastoma cells induces both neurite outgrowth and phosphorylation of microtubule-associated protein MAP-1B

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Summary

The addition of two synthetic peptides with antiprotease activity to the culture medium of mouse neuroblastoma cells results in the promotion of neurite outgrowth. One of these peptides has a sequence corresponding to the reactive center of protease nexin-1 and inhibits both trypsin and thrombin. Its effect on neuroblastoma cells is similar to that found on serum withdrawal from the culture medium, giving rise to cells with one or two long neurites, and is reversed upon the addition of thrombin to the culture medium. The sequence of the other peptide is present in one of the precursor proteins of the main component of the amyloid plaques of Alzheimer's disease patients' brains, and corresponds to protease nexin-2. It can inhibit trypsin but fails to inhibit thrombin at low doses. Its effect on neuroblastoma cells is slightly different from that observed after serum deprivation, as a significant proportion of stellate cells, with short and branched neurites, is observed. An increase in the phosphorylation of microtubule-associated protein MAP-1B, which accompanies neurite outgrowth induced by serum deprivation, is also observed upon addition of the two antiprotease synthetic peptides, although the nexin-2 (amyloid) peptide induces a less marked increase in phosphorylated MAP-1B than does the nexin-1 peptide. These results may be correlated with the different antiprotease activities of both synthetic peptides, thus suggesting a role for a balance between trypsin-like and thrombin-like proteases and their inhibitors in eliciting neurite outgrowth under normal and pathological conditions.

Key words: neuroblastoma cells, neuronal morphogenesis, protease inhibitor, nexin, β amyloid protein, microtubule protein phosphorylation, Alzheimer's disease.

Introduction

Neuroblastoma cells have been widely used as a model system for studying the early stages of neuronal differentiation, as they can extend axon-like processes (neurites). Neurite extension occurs in response to extracellular signals such as the addition of dibutyryl cyclic AMP (Fumarski *et al.* 1971) or prostaglandins (Prasad, 1972, 1975), or the withdrawal of serum from the culture medium (Eddé *et al.* 1987; Gard and Kirschner, 1985; Grand *et al.* 1989; Seeds *et al.* 1970). Thus, neurite outgrowth may be regulated by an interplay between positive and negative signals present in the extracellular medium. As a consequence of a cell receiving extracellular signals, a cascade of several intracellular events, including protein phosphorylation, may take place as a prelude to neurite extension. However, little is known about the factors that govern neurite outgrowth and the pathways of signal transduction that are implicated.

From observations of the effect of serum deprivation on neurite extension, the presence of neurite outgrowth inhibitory factors in serum has been proposed. Recently, it has been suggested that the serum factors responsible for inhibition of neurite extension are serine proteases (primarily thrombin) (Cunningham and Gurwitz, 1989; Gurwitz and Cunningham, 1988, 1990; Grand *et al.* 1989). The addition of thrombin to differentiated neuroblastoma cells reverses neurite outgrowth (Cunningham and Gurwitz, 1989; Gurwitz and Cunningham, 1988, 1990). In addition to its inhibitory effect on neuroblastoma cell differentiation, thrombin also reverses neurite outgrowth (induced spontaneously, by serum deprivation or dibutyryl cyclic AMP treatment) in other neuroepithelial cells, including primary cultures from human fetal cerebellum and hippocampus (Grand *et al.* 1989). Furthermore, thrombin stimulates the mitosis of neuroepithelial cell lines (Grand *et al.* 1989).

These results raise the possibility that proteases may serve as mitogens for neuroblasts in the developing brain, although it is not known whether this putative role is performed by serum proteases, which may be present in brain before the full establishment of the blood–brain barrier, or brain-specific endogenous serine proteases. The presence of mRNA for prothrombin in human brain has been reported (Cunningham and Gurwitz, 1989), and plasminogen activators have also been identified in brain (Krystosek and Seeds, 1981*a*,*b*; 1984; Soreq and Miskin, 1981, 1983; Verall and Seeds, 1988; Pittman *et al.* 1989). According to this view, the cessation of mitosis and the morphological differentiation of neuroblasts into neurons

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would require the inhibition of proteases, which might be mediated through protease inhibitors released by glial cells. Supporting this possibility is the fact that a glia-derived neurotrophic factor that induces neurite extension in cultures *in vitro* is a serine protease inhibitor referred to as glia-derived nexin (GDN), identical to protease nexin-1 (PN-1) (Gloor *et al.* 1986; Monard, 1988; Stone *et al.* 1987). The neurite outgrowth-promoting effect of GDN is blocked by serine proteases such as thrombin and urokinase (Monard, 1988). In addition to GDN, other protease inhibitors derived from glial-related cells have been described. For example, a plasminogen activator inhibitor isolated from glioblastoma is also able to induce neurite outgrowth (Rehemtulla *et al.* 1988).

In this work, we have analyzed the effect of two different protease inhibitors on mouse neuroblastoma cell differentiation, studying the phosphorylation of microtubule protein that accompanies neurite extension and may serve as an early marker for neuronal differentiation (Aletta *et al.* 1988; Eddé *et al.* 1987; Gard and Kirschner, 1985; Díaz-Nido *et al.* 1988). One of the peptide protease inhibitors that we have used is a synthetic peptide containing the sequence corresponding to the reactive center of GDN (PN-1), and the other is a synthetic peptide with a sequence similar to that found in the reactive center of the Kunitz family of protease inhibitors (Carrell, 1988; Laskowski and Kato, 1980). The latter peptide has a sequence that is present in an isoform of protein A4 (also referred to as β amyloid protein), which is the precursor of the principal component of the amyloid deposits found in Alzheimer's disease patients' brains (Kitaguchi *et al.* 1988; Ponte *et al.* 1988; Sinha *et al.* 1990; Tanzi *et al.* 1988) and is identical to protease nexin 2 (PN-2) (Oltersdorf *et al.* 1989; Van Nostrand *et al.* 1989).

Materials and methods

Materials

Thrombin (EC 3.4.21.5.; 120 units mg^{-1}) was purchased from Boehringer Mannheim (Cat. no. 602400). Soybean trypsin inhibitor was obtained from Sigma. [^{32}P]phosphate was from Amersham International. Peptide 1, referred to as nexin-1 peptide, contains the sequence CARSSP (Cys-Ala-Arg-Ser-Ser-Pro), which is found in the reactive center of GDN (PN-1) (Gloor *et al.* 1986). Peptide 2, referred to as the amyloid peptide, contains the sequence CRAMI (Cys-Arg-Ala-Met-Ile), which is found in the isoform of protein A4, a precursor of the main component of the amyloid deposits found in Alzheimer's disease patients' brains, which corresponds to PN-2 (Carrell, 1988; Kitaguchi *et al.* 1988; Oltersdorf *et al.* 1989; Ponte *et al.* 1988; Sinha *et al.* 1990; Tanzi *et al.* 1988). Peptide 3 contains the sequence SPAKSPSLSPS, similar to that of peptide 1. Peptides were synthesized on an automatic solid-phase peptide synthesizer (type 430A, Applied Biosystems) and purified by reverse-phase HPLC on a NOVAPAK C-18 column. They were lyophilyzed, weighed and dissolved (1 mg ml^{-1}) in phosphate-buffered saline (10 mM sodium phosphate, pH 7.2, 140 mM NaCl).

Cell culture and labeling

N2A mouse neuroblastoma cells (ATCC CCL 131, American Type Culture Collection, Rockville, MD), seeded at 10^4 cells $well^{-1}$ on 24-well microtiter plates, were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum, and induced to differentiate either by transferring to DME without serum or by adding from 10 to 50 $\mu g\,ml^{-1}$ of nexin-1 or amyloid synthetic peptides; or 300 ng ml^{-1} of yeast recombinant rat GDN (HQSS-Y-30-22; a generous gift from Dr Denis Monard, Friedrich Miescher-Institut, Basel, Switzerland). N2A cells were labelled as described previously following overnight incubation of the cell culture with 1 mCi ml^{-1} of $^{32}PO_4$ (HCl- and carrier-free) (Díaz-Nido *et al.* 1988).

Immunoprecipitation of MAP-1B from labelled cell extracts was performed as previously described (Díaz-Nido *et al.* 1988), using a monospecific polyclonal antibody to MAP-1B and collecting antigen–antibody complexes by the addition of a *Staphylococcus aureus* suspension. Immunoprecipitates were collected by centrifugation, resuspended in 1% SDS and characterized by gel electrophoresis and autoradiography.

Immunofluorescence microscopy

N2A cells grown on glass coverslips and induced to differentiate were fixed for 10 min with cold methanol. The cells were then washed with PBS, followed by a 1 h incubation with a monoclonal antibody to β-tubulin (diluted 1:1000 in PBS). They were then washed with PBS, incubated for 30 min with fluorescein-conjugated goat anti-mouse IgGs, washed again with PBS and the coverslips mounted on glass slides using glycerol (90% in PBS).

Protease assay

Trypsin (10 $\mu g\,ml^{-1}$) or thrombin (10 $\mu g\,ml^{-1}$) was preincubated with various concentrations of the inhibitors to be tested in 1 ml of 100 mM Tris–HCl, pH 7.5, containing bovine serum albumin (1 mg ml^{-1}) at room temperature for 15 min. Residual activity was then assayed by continuous spectrophotometric recording (405 nm) upon adding 10 μl of either 1 mM chromozym TRY (carbobenzoxy-Val-Gly-Arg-*p*-nitroanilide acetate) or 1 mM chromozym TH (tosyl-glycyl-prolyl-arginyl-*p*-nitroanilide acetate) (chromozym TRY and TH were purchased from Boehringer Mannheim).

Results

Inhibition of protease activity by synthetic peptides

Two synthetic peptides have been used throughout this study, one containing the reactive centre of nexin-1 (Gloor *et al.* 1986), which is known to be a serine protease inhibitor (Stone *et al.* 1987), and another with the sequence of the protein precursor (A4) of amyloid deposits of Alzheimer's disease patients' brains, which has been considered a putative protease inhibitor and neurotrophic factor (Carrell, 1988; Kitaguchi *et al.* 1988). The plot of trypsin and thrombin inhibition by the synthetic peptides indicates different protease inhibitory properties for each of them (Fig. 1). Whereas the nexin-1 peptide is an inhibitor of both trypsin and thrombin, the amyloid peptide at low doses seems to be effective only as a trypsin inhibitor. A third peptide (peptide 3, described in Materials and methods), containing a sequence related to that of nexin peptide, was also tested. Fig. 1 indicates that, essentially, the peptide does not inhibit serine proteases. However, when glia-derived nexin was tested a strong protease inhibition was found (Fig. 1).

Although synthetic peptides are notably poorer protease inhibitors than the native proteins from which they are derived – indicating a role for protein conformation in the inhibition of proteolysis – their enzyme specificities correspond to those reported for the native proteins: PN-1 is a general inhibitor of serine proteases including trypsin, thrombin and plasminogen activators (Stone *et al.* 1987), and the Alzheimer's β-amyloid precursor protein is a relatively specific inhibitor of a few trypsin-like proteases including trypsin and plasminogen activators (Sinha *et al.* 1990).

Addition of protease inhibitors to the culture medium of neuroblastoma cells induces neurite outgrowth

Mouse neuroblastoma cells develop neurites upon serum

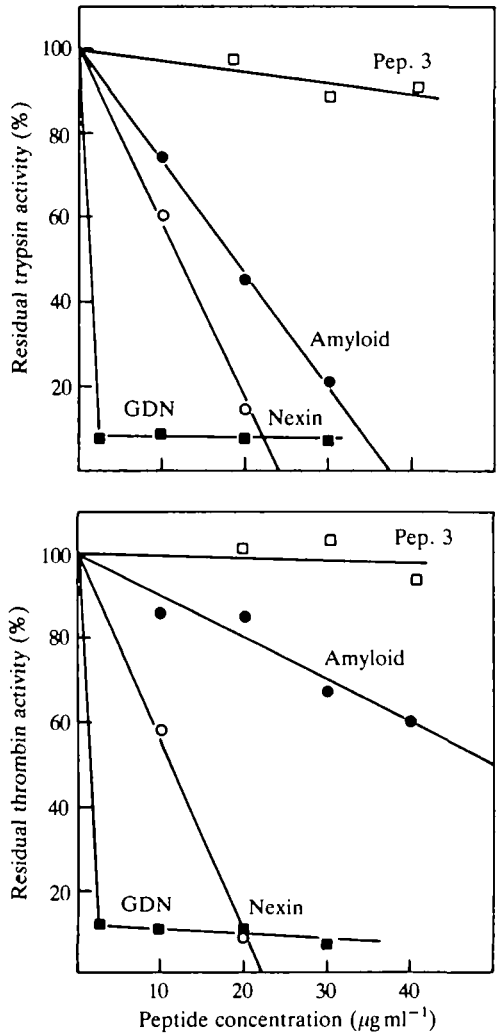

Fig. 1. Inhibition of protease activity by synthetic peptides and glia-derived nexin. Residual protease activity of trypsin and thrombin preincubated with synthetic peptides was measured as indicated in Materials and methods using chromozym TRY and chromozym TH as chromogenic substrates. Peptide concentrations are derived from the weight of the lyophilized peptides and should not be considered as expressing the true concentration of active peptide. The effects of peptide 3 (□) (see Materials and methods); amyloid peptide (●), nexin peptide (○) and glia-derived nexin (■) are indicated.

withdrawal (Eddé *et al.* 1987; Gard and Kirschner, 1985; Seeds *et al.* 1970) and it has been suggested that this is due to the removal of serum serine proteases (Gurwitz and Cunningham, 1988). To test this possibility further, we have added synthetic nexin-1 and amyloid peptides to the culture medium of neuroblastoma cells in the presence of serum. Fig. 2 indicates that the addition of synthetic peptides results in neurite outgrowth in neuroblastoma cells to the same extent as that observed upon serum removal or upon the addition of glia-derived nexin.

Fig. 2 also shows that there are morphological differences between the cells incubated in the presence of nexin-1 or amyloid peptides. Neuroblastoma cells differentiated in the presence of nexin-1 peptide are indistinguishable from cells differentiated upon either GDN addition or serum deprivation. These cells are mainly unipolar or bipolar, i.e. they are characterized by one or two long neuritic processes (Fig. 2A). However, when neuroblastoma cells are induced to differentiate upon addition of the amyloid peptide, a significant proportion (37.5 %) of cells are multipolar, i.e. they become stellate cells bearing several shorter neurites (Fig. 2A). Neurites from stellate cells are also clearly more branched than those from unipolar or bipolar cells. Antibodies to tubulin, the major component of the neurite microtubule cytoskeleton, stain neurites from both stellate and unipolar neuroblastoma cells equally well when analyzed by indirect immunofluorescence (Fig. 2B), although the smaller neurite branches are less intensely stained than the main neurites in stellate cells. No morphological differences were observed when peptide 3 was added to the culture medium.

Addition of protease inhibitors to the culture medium of neuroblastoma cells increases the phosphorylation of microtubule proteins

Phosphorylation of microtubule proteins, mainly that of the microtubule-associated protein MAP-1B, is coupled to neurite outgrowth in a variety of cell lines of neural origin (Aletta *et al.* 1988; Díaz-Nido *et al.* 1988; Gard and Kirschner, 1985).

In view of the fact that the amyloid peptide induces a neuroblastoma cell differentiation that differs from that observed upon either nexin-1 peptide addition or serum withdrawal, we have studied the phosphorylation of microtubule-associated protein MAP-1B under these conditions.

Fig. 3 shows that the addition of amyloid peptide to the culture medium of neuroblastoma cells induces an increase in the phosphorylation of MAP-1B, as compared with cells growing in medium without the amyloid peptide. However, the increase in MAP-1B phosphorylation is not as large as that found upon serum withdrawal.

Gurwitz and Cunningham (1988) have indicated that neurite retraction could occur upon thrombin or serum addition to the culture medium. To test whether neurite retraction is correlated with a dephosphorylation of MAP-1B, pure thrombin was added to a serum-free culture medium, resulting in the lowering of the level of phosphorylated MAP-1B to a value close to that found in cells growing in serum-containing medium. When serum was added to differentiated neuroblastoma cells, a decrease in MAP-1B-associated radioactive counts was also found. As a control for the previous experiment, incubation with colcemid was also performed. In the presence of this compound, which prevents microtubule assembly, neurites were retracted, but no significant decrease in MAP-1B phosphorylation was found, as previously described (Díaz-Nido *et al.* 1988; Gard and Kirschner, 1985).

These results clearly indicate that only in the presence of protease inhibitors (or in the absence of active proteases) does MAP-1B phosphorylation take place in a manner coupled to neurite extension.

Table 1 shows the correlation between the level of phosphorylated MAP-1B, the percentage of differentiated cells and mean neurite length.

Discussion

A correlation between the ability to inhibit proteases and the neurite extension-promoting ability of the synthetic peptides used in this work has been established. The addition of nexin-1 synthetic peptide to the culture medium of neuroblastoma cells results in neuroblastoma cell differentiation similar to that caused by addition of

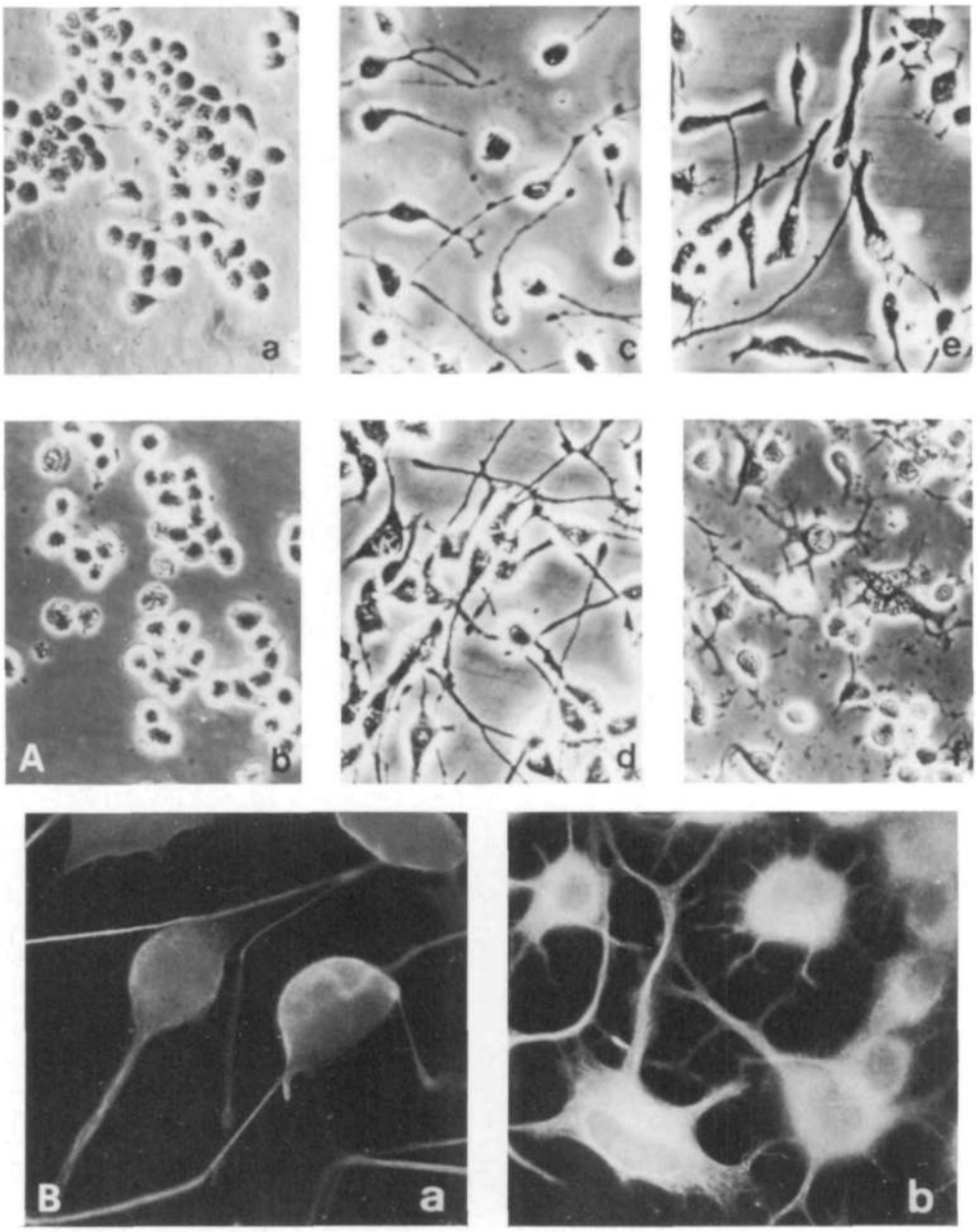

Fig. 2. Promotion of neurite growth in mouse neuroblastoma cells by protease inhibitory peptides. (A) N2A cells (10^4 cells well^{-1}) were grown in the presence of 10% fetal calf serum without any addition (a), in the presence of 30 μg ml^{-1} peptide 3 (b), or maintained in the absence of serum for 24 h (c), grown in the presence of 10% fetal calf serum supplemented with 30 ng ml^{-1} of glia-derived factor for 24 h (d), or in the presence of 10% fetal calf serum supplemented with 30 μg ml^{-1} of nexin peptide for 24 h (e), or in the presence of 10% fetal calf serum supplemented with 30 mg ml^{-1} of amyloid peptide (f). (B) Immunofluorescence microscopy of N2A cells differentiated by addition of either nexin-1 peptide (a) or amyloid peptide (b) stained with an anti-tubulin antibody as described in Materials and methods.

pure GDN (PN-1) or withdrawal of serum from the culture medium. This effect seems to be correlated with the inhibition of thrombin-like proteases, as it is reversed by the addition of pure thrombin, in agreement with results previously obtained in other laboratories (Cunningham and Gurwitz, 1989; Gurwitz and Cunningham, 1988, 1990; Grand *et al.* 1989). Differentiated neuroblastoma cells obtained upon inhibition of thrombin-like proteases are primarily unipolar or bipolar.

The addition of amyloid synthetic peptide to the culture medium of neuroblastoma cells also results in neuroblastoma cell differentiation, but in this case, a substantial proportion of multipolar cells is observed. This may be correlated with the ability of amyloid peptide to inhibit trypsin-like proteases and its inability to inhibit thrombin-like proteases. Multipolar cells are often found transiently during neuroblastoma cell differentiation induced by serum withdrawal (or addition of nexin-1 peptide), although they eventually become unipolar or bipolar. It is tempting to speculate that the inhibition of trypsin-like proteases, possibly located on the cell surface of neuroblastoma cells, is sufficient to promote neurite outgrowth, while full neurite development requires the additional inhibition of serum thrombin. This latter step is

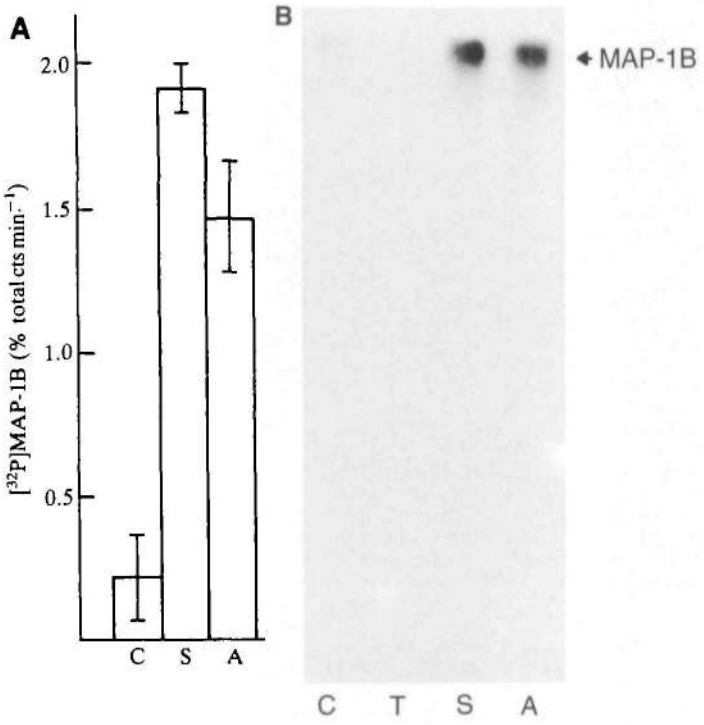

Fig. 3. Stimulation of MAP-1B phosphorylation in neuroblastoma cells by protease inhibitory peptides. (A) The amounts of phosphorylated MAP-1B estimated by immunoprecipitation from labeled extracts of cells grown in the presence of 10% fetal calf serum (C), in the absence of serum (S) or in the presence of 10% fetal calf serum and 30 $\mu g\,ml^{-1}$ amyloid synthetic peptide (A). The histograms correspond to the mean values of three experiments, error bars represent the standard deviations. (B) The autoradiography after gel electrophoresis of the immunoprecipitates containing phosphorylated MAP-1B obtained from cells grown in the presence of 10% fetal calf serum (C), in the presence of 10 $\mu g\,ml^{-1}$ thrombin in serum-free medium (T), in serum-free medium (S), and in the presence of 10% fetal calf serum and 30 $\mu g\,ml^{-1}$ amyloid peptide (A).

observed upon either serum withdrawal or addition of thrombin inhibitors, but is not found with amyloid peptide, which cannot inhibit thrombin. The identity of the putative cell surface trypsin-like proteases possibly involved in the initiation of neurite extension remains to be established, although there is some evidence showing the association of plasminogen activators, particularly tissue plasminogen activator with the membrane of neural cells (Krystosek and Seeds, 1981*a*,*b*, 1984; Soreq and Miskin, 1981, 1983; Verrall and Seeds, 1988; Pittman *et al.* 1989). The putative role of plasminogen activators in neural morphogenesis is also supported by the fact that a plasminogen activator inhibitor derived from glioblastoma cells is able to induce neurite outgrowth (Rehemtulla *et al.* 1988).

The addition of amyloid peptide to the culture medium of neuroblastoma cells, with the subsequent inhibition of cell surface trypsin-like proteases (possibly plasminogen activators) together with the maintained activity of serum thrombin, may also provide some clues about the growth of neurites in certain pathological conditions. Thus, an imbalance in proteolysis might be involved in the etiology of Alzheimer's disease, as suggested by Wagner *et al.* (1989). The aberrant processing of the β protein in

Table 1. *Correlation between MAP-1B phosphorylation and neurite extension*

Culture conditions	Phosphorylated MAP-1B (% of maximum value)	Neurite-bearing cells (% of cells)	Mean neurite length (μm)
DME+FCS	11±8	25±17	25±20
DME−FCS	100±4	88±17	307±75
DME−FCS+T	9±6	15±12	15±20
DME−FCS+FCS	10±7	17±12	18±20
DME+FCS+NP	97±5	86±15	300±72
DME+FCS+AP	75±10	75±25	199±130

N2A cells were grown in culture medium containing fetal calf serum (DME+FCS), in culture medium lacking serum (DME−FCS), in culture medium lacking serum but supplemented with thrombin (DME−FCS+T), in culture medium lacking serum but supplemented with serum again, once the cells were morphologically differentiated (DME−FCS+FCS), in culture medium containing serum and 30 $\mu g\,ml^{-1}$ of nexin-1 peptide (DME+FCS+NP) or in culture medium containing serum and 30 $\mu g\,ml^{-1}$ of amyloid peptide (DME+FCS+AP), as indicated in Materials and methods. The level of phosphorylated MAP-1B was determined after immunoprecipitation and expressed as percentage of the maximum value. Data indicate mean values from three experiments and standard deviations.

Alzheimer's disease with its deposition in amyloid plaques, together with the observed decrease in protease nexin-1 (Wagner *et al.* 1989) would lead to an inhibition of trypsin-like proteases (possibly plasminogen activators), as well as augmented thrombin-like protease activity in the surroundings of amyloid plaques.

The mechanisms by which the addition of protease inhibitors to the culture medium promotes neurite extension are not known (Monard, 1988). Nothing is known about the existence of cell surface receptors for extracellular protease inhibitors, although it has been proposed that membrane-associated proteases could perform this role (Monard, 1988). It is also not known whether the binding of protease inhibitors to membrane-associated proteases triggers signal transduction events similar to those observed when growth factors bind to their cell surface receptors. Alternatively, the reduction in cell-surface proteolytic activity may have an effect in increasing the density and stability of adhesion molecules, thus allowing stronger cell attachment, as indicated above. The binding of adhesion molecules to the substratum may, in turn, trigger a signal transduction pathway from the cell surface to the cytoplasm. The recently reported influence of neural cell adhesion molecules on second messenger systems may support this hypothesis (Schuch *et al.* 1989). It includes a decrease in phosphoinositide turnover, a decrease in the activity of protein kinase C, an increase in the free cytoplasmic calcium concentration and a decrease in intracellular pH (Schuch *et al.* 1989).

These intracellular events may lead both to an increase in the phosphorylation of microtubule-associated protein MAP-1B, which is largely due to the activation of a casein kinase II-related enzyme (Díaz-Nido *et al.* 1988), and to the promotion of the assembly of the microtubules that constitute the inner scaffolding of the developing neurite. The fact that colcemid inhibits microtubule assembly, and consequently blocks neurite outgrowth, without affecting the increase in phosphorylated MAP-1B (Gard and Kirschner, 1985; Díaz-Nido *et al.* 1988), supports the view that the activation of the enzyme responsible for MAP-1B phosphorylation is an early event in the process of neurite outgrowth (Díaz-Nido *et al.* 1988). Thus, the activation of a casein kinase II-type MAP-1B kinase in response to

extracellular protease inhibitors may actually be the consequence of modifications on second messenger systems resulting from the increased attachment of neural adhesion molecules to the extracellular matrix, which occurs after cell surface protease inhibition.

Interestingly, it has been indicated that neurite extension promoted by the addition of nerve growth factor (NGF) in rat pheochromocytoma cells (PC12) also correlates well with the phosphorylation of MAP-1B (Aletta *et al.* 1988). However, it is not known which kinase is involved in this post-translational modification, since several protein kinase activities increase during the differentiation of PC12 cells (Mutoh *et al.* 1988). Perhaps the activation of the casein kinase II-related enzyme that phosphorylates MAP-1B is a common point in different signal transduction pathways that result in neurite extension. Thus, NGF or adhesion molecules may trigger different cascades of intracellular events that finally give rise to the activation of several protein kinases, including the enzyme responsible for MAP-1B phosphorylation.

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