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MAP-1B/TAU functional redundancy during laminin-enhanced axonal growth

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SUMMARY

When cultured cerebellar macroneurons develop attached to a laminin-containing substrate or after the acute addition of laminin to the tissue culture medium, there is an acceleration in the rate and extent of axonal elongation. Furthermore, laminin is capable of inducing axonal formation and microtubule stabilization in neurons arrested at stage II of neuritic development by tau suppression (Caceres and Kosik, 1990; Caceres et al., 1991). Laminin-enhanced or induced axonal extension is paralleled by a selective and dramatic incorporation of phosphorylated MAP-1b into axonal microtubules. Axonal formation in neurons growing in the presence of laminin is prevented by treatment of the cultures with a mixture of MAP-1b and tau antisense oligonucleotides, but not by the single suppression of any one of these MAPs. However, suppression of MAP-1b, but not of tau, greatly reduces the increase in the rate and extent of axonal elongation induced by laminin. No such effects are elicited by MAP-1b antisense oligonucleotides in neurons growing in the absence of laminin, e.g. polylysine alone, where most of the MAP-1b present in the cells is dephosphorylated and not associated with the cytoskeleton. Taken collectively, these data suggest that, with regard to axonal elongation, MAP-1b and tau can be functionally substituted, and that extracellular matrix molecules, such as laminin, affect axonal extension by promoting the in vivo utilization of MAP-1b.

Key words: Laminin, Axon, Polarity, Microtubule stabilization, Cultured neuron, Microtubule-associated protein

INTRODUCTION

It is now well established that laminin, an extracellular matrix glycoprotein, can significantly influence the rate and direction of neurite growth in both central and peripheral neurons (Sanes, 1989). The idea that laminin has a role in the regulation of neurite extension is supported by the fact that it is expressed at stages critical for terminal neuronal differentiation in situ (Gordon-Weeks et al., 1988; Hagg et al., 1989; Osterhout et al., 1992; Stewart and Pearlman, 1987) and that it promotes neurite outgrowth in cultured neurons (Buettner and Pittman, 1991; Manthorpe et al., 1983; Rogers et al., 1983). Besides, several recent reports have raised the possibility that laminin, as well as other extracellular matrix molecules (ECM), may have an important modulatory function in the establishment of neuronal polarity; thus, in both cultured hippocampal pyramidal cells and in sympathetic neurons, laminin selectively enhances axonal outgrowth and the development of polarity (Chamack and Prochiantz, 1988; Lafont et al., 1982; Lein et al., 1992; Lein and Higgins, 1989; Lochter and Schachner, 1993; Osterhout et al., 1992). The intracellular molecular events underlying these responses are currently unknown.

Microtubules are prominent elements of the neuronal cytoskeleton which are involved in the growth and maintenance of neurites (Mitchison and Kirschner, 1988). Because microtubule formation is one of the key events mediating neurite elongation (Cleveland and Hoffmann, 1991), it may well be that laminin exerts its action by regulating the expression or the activity of microtubule-associated proteins (MAPs) known to promote tubulin assembly and microtubule stability during active process extension (Brugg et al., 1993; Caceres and Kosik, 1990; Caceres et al., 1991, 1992; Esmaeli-Azad et al., 1994; Hanemaaijer and Ginzburg, 1991; Harada et al., 1994). To test this hypothesis we have suppressed the expression of MAP-1b and/or tau in cerebellar macroneurons cultured in the presence of polylysine or laminin and examined its consequences on axonal formation. The results obtained indicate that: (1) laminin is capable of stimulating axonal elongation in cerebellar macroneurons, an event paralleled by a dramatic incorporation of MAP-1b into the cytoskeleton; and (2) depending on the substrate in which neurons develop they may use cooperatively or alternatively MAP-1b and/or tau for sustaining axonal elongation and microtubule stabilization.

MATERIALS AND METHODS

Cell cultures

Dissociated cultures of cerebellar macroneurons were prepared from

15-day-old rat embryos as described previously (Caceres et al., 1992; Ferreira et al., 1989). Cells were plated onto polylysine-coated glass coverslips at densities ranging from 5,000 to 15,000 cells/cm^2 and maintained with Dulbecco's modified Eagle's medium (DMEM) plus 10% horse serum for 1 hour. The coverslips with the attached cells were then transferred to 60 mm Petri dishes containing serum free medium plus the N2 mixture of Bottenstein and Sato (1979). All cultures were maintained in a humidified, 37°C incubator with 5% CO_2.

To bind laminin to the substrate, polylysine coated coverslips were soaked in DMEM containing mouse EHS laminin (Gibco, Sigma Chemical Co., or Boehringer Mannheim) at a concentration of 10 μg/ml (unless otherwise specified) overnight at 4°C. Laminin from all three sources was found to have the same effects on the neurite outgrowth response of cerebellar macroneurons. In some experiments, laminin was directly added to the culture medium from a 1 mg/ml stock solution to make a final concentration of 20 μg/ml.

Antisense oligonucleotides

Two non-overlapping MAP-1b antisense oligonucleotides, designated As1 or As2, identical to those employed by Brugg et al. (1993) to block MAP-1b expression in PC 12 cells were used at 25 μM or 50 μM in most of the experiments. These antisense oligonucleotides were based on the sequence of a clone isolated from a post-natal day 5 rat cDNA library (Garner et al., 1990). A third antisense MAP-1b oligonucleotide, designated As3 corresponding to mouse MAP-1b complementary cDNA sequence (Noble et al., 1989) from positions −11 to +14 was also used in some experiments. Inhibition of tau expression was performed with the antisense oligonucleotide RT 11/14 as described previously (Caceres and Kosik, 1990; Caceres et al., 1991, 1992). The sequences selected were not found in the data base in any other known sequence. Control cultures were treated with the same concentration of the corresponding sense-strand oligonucleotide. The oligonucleotides were synthesized on an Applied Biosystem synthesizer, purified by reverse chromatography, and taken up in serum free medium. The oligonucleotides (sense or antisense) were added to the culture medium 1 hour after cell plating and again at 12 hour intervals until fixation.

Immunofluorescence

Cells were fixed before or after detergent extraction under microtubule-stabilizing conditions and processed for immunofluorescence as previously described (Caceres et al., 1992; DiTella et al., 1994; see also Brown et al., 1992). The primary antibodies used were: a monoclonal antibody (mAb) against all isoforms of α-tubulin (clone DM1A, mouse IgG, Sigma Chemical Co.) diluted 1:100; a mAb against tyrosinated α-tubulin (clone TUB-1A2, mouse IgG, Sigma Chemical Co.) diluted 1:2,000; a rabbit antiserum against total tubulin (Sigma Chemical Co.) diluted 1/100; an affinity purified rabbit polyclonal antibody against detyrosinated α-tubulin diluted 1:1,000 (DiTella et al., 1994); tau mAbs against non-phosphorylated epitopes of these molecules (clones tau-1 and 5E2) (Caceres et al., 1992) diluted 1:50; MAP-2 mAbs (clones AP-14 and AP-18) (Caceres et al., 1992) diluted 1:100; a MAP-1a mAb (Sigma Chemical Co.) diluted 1:50; a mAb against acetylated α-tubulin (clone 6-11B-1) (Ferreira and Caceres, 1989). We also used several antibodies against MAP-1b: mAb AA6, which recognizes a conserved non-phosphorylated and non-phosphorylatable epitope on MAP-1b (Sigma Chemical Co.) (Brugg et al., 1993) diluted 1:50; mAb 150, which recognizes a phosphorylated epitope (Ulloa et al., 1993a,b, 1994) diluted 1:200, and rabbit antiserum 531, which recognizes a phosphorylatable epitope when it is dephosphorylated (Ulloa et al., 1993a,b, 1994). Secondary antibodies were goat anti-rabbit IgG rhodamine conjugated (Boehringer Mannheim Corp.) and goat anti-mouse IgG fluorescein or rhodamine labeled (Boehringer Mannheim Corp.).

For some experiments the relative intensity of MAP-1b immunofluorescence was evaluated in fixed unextracted cells or in detergent-extracted cytoskeletons double labeled with mAb 150 and rabbit antiserum 531 using quantitative fluorescence techniques as described previously (Brown et al., 1992; DiTella et al., 1994). To image labeled cells, the incoming epifluorescence illumination was attenuated with glass neutral density filters. Images were formed on the faceplate of a Silicon Intensified Target camera (SIT; Hamamatsu Corp., Middlesex, NJ), set for manual sensitivity, gain and black level. They were digitized directly into a Metamorph/Metafluor Image Processor (Universal Imaging Corporation, West Chester, PA) controlled by a host IBM-AT computer. Fluorescence intensity measurements were performed pixel by pixel along the longitudinal axis of identified neurons. Using this data, we then calculated the average fluorescence intensity within the cell body, inner, middle and distal third of identified neurites (either minor neurites or axons). Background levels were those detected in unlabeled cells.

Morphometric analysis of neuronal shape parameters

Images were digitized on a video monitor using JAVA software (JAVA: Jandel Video Analysis Software, Jandel Scientific, Corte Madeira, CA). To measure neurite length, fixed unstained or antibody-labeled cells were randomly selected and traced from a video screen using the morphometric menu of the image processor (Caceres et al., 1992). A total of at least a 100 cells was evaluated for each experimental condition and time point; differences among groups were analyzed by the use of ANOVA and Student-Newman Keuls test.

Quantification of microtubule protein levels

Whole cell homogenates or detergent-extracted cytoskeletons from cell cultures, as well as microtubules from young or adult rats were prepared as described previously (Caceres et al., 1992; Ferreira et al., 1990; Ferreira and Caceres, 1989) and the levels of microtubular proteins determined by dot immunobinding using the antibodies described above and ^{125}I labeled Protein A following previously reported procedures (Caceres et al., 1988, 1992; Ferreira and Caceres, 1989). Standard curves were constructed and the amount of MAP-1b, MAP-2 and tau in unknown samples was determined by extrapolation of the linear regions of the unknown to the linear portions of the standard. In addition, MAP-1b or tau protein levels were measured by quantitative immunoblotting as described by Drubin et al. (1985). For such a purpose, immunoblots were probed with the mAb AA6 or tau-1, then reacted with a rabbit antimouse IgG diluted 1:1,000 (Boehringer Mannheim Corp.) and finally incubated with iodinated Protein A. Autoradiography was performed on Kodak X-omat AR film using intensifying screens. Autoradiographs were aligned with immunoblots and MAP-1b and tau levels were quantitated by scintillation counting of nitrocellulose blot slices. It is worth mentioning that the values of MAP-1b or tau determined by dot immunobinding assays or quantitative western blotting gave almost identical results.

In addition, for each set of samples analyzed by quantitative immunoblotting, a duplicate set was also reacted with the mAb AA6 or tau-1 and developed using a mouse ProtoBlot Western Blot Alkaline Phosphatase System (Promega Corporation, Madison, WI).

RESULTS

Axonal elongation in cerebellar macroneurons cultured in the presence of laminin

Cultured cerebellar macroneurons have been extensively used to study the dynamics of neurite growth and differentiation, as well as the expression and function of MAPs during neuronal morphogenesis (Caceres and Kosik, 1990; Caceres et al., 1991, 1992; DiTella et al., 1994; Ferreira et al., 1987, 1989; Ferreira and Caceres, 1989). All these in vitro studies have used homopolymers such as polylysine as an adhesive substrate;

under this condition cells extend neurites following a quite typical sequence of morphological transformations (Caceres and Kosik, 1990; Caceres et al., 1991, 1992). To assess the effect of laminin on neurite outgrowth of cerebellar macroneurons, we first examined cultures at stage III of neuritic development when neurons had become polarized and the axon could be readily distinguished from the minor processes. Forty-eight hours after plating, virtually all cerebellar macroneurons display one long axon and 3 to 4 minor neurites; both types of processes have few collateral branches. Substrate-bound laminin (10 or 20 μg/ml) causes a significant increase in axonal length and collateral branching without modifying the number, length or branching pattern of minor neurites (Tables 1 and 2); laminin also induces a more rapid onset of axonal elaboration and increased rate of axonal elongation (Fig. 1a). Thus, after 8 hours in vitro more than 80% of neurons growing on a laminin-containing substrate display a single thin neurite of uniform caliber and a mean length of more than 100 μm; the tips of these neurites are highly immunoreactive for synapsin I and the growth-associated protein GAP-43 (data not shown). By contrast, laminin has little or no effect on the number, rate of growth, and time course of appearance of minor processes.

As previously reported in other cell systems (Chamack and Prochiantz, 1988; Rivas et al., 1992) laminin also causes rapid axonal elongation when it is acutely added to the culture medium of cerebellar macroneurons growing on polylysine. Under our experimental conditions this effect is detected between 4-6 hours after the addition of laminin (20 μg/ml) and persists for at least 24 hours, the longest time period analyzed (Table 3).

The expression of MAP-1b and tau in neurons growing on laminin

Dot immunobinding assays (Fig. 1b) and western blot analysis (Fig. 2a) of whole cells homogenates reacted with the mAb AA6 (Brugg et al., 1993) revealed that embryonic (E 15) cerebellar cells express MAP-1b at the time of plating (see also (Caceres et al., 1992). In cerebellar macroneurons growing on polylysine MAP-1b protein levels remain high during the initial phases of neurite outgrowth and axonal elongation. Fig. 1b also shows that during the first 24 hours in culture most of the MAP-1b present in these cells is not associated with microtubules since it is extracted with the Triton-soluble fraction under microtubule stabilizing conditions; after that time period there is a progressive incorporation of the protein into microtubules. No changes in the induction profile of MAP-1b are detected in neurons maintained on a laminin-containing substrate when compared with control ones (Fig. 1b). However, on laminin a rapid and dramatic incorporation of MAP-1b into the Triton insoluble microtubule fraction closely paralleled axonal outgrowth (Fig. 1b).

Since previous studies have suggested that MAP-1b phosphorylation increases the interaction of MAP-1b to microtubules we sought to determine whether laminin-stimulated axonal growth is paralleled by an enhanced expression and incorporation into microtubules of phosphorylated MAP-1b. Two major modes of MAP-1b phosphorylation have been described which can be distinguished by the use of antibodies to phosphorylation sensitive epitopes (Ulloa et al., 1993a,b, 1994): one in which a proline directed protein kinase (PDPK) appears to be involved (mode I), and one which is presumably catalyzed by casein kinase II (mode II). We used two different antibodies against MAP-1b for this experiment; one of them, mAb 150, recognizes a phosphorylated mode I epitope, while the other one, rabbit antiserum 531, recognizes a phosphorylatable mode I site when it is dephosphorylated.

In cerebellar macroneurons maintained on a laminin-containing substrate an intense and selective staining of axonal processes is detected with the mAb 150. This staining is more prominent at the distal end of the growing processes, including their growth cones, and present in cells fixed before or after detergent extraction. Minor processes do not label with the

Table 1. Effect of laminin on process outgrowth by cerebellar macroneurons in 48 hour cultures

Group	Total neuritic length/neuron	Axonal length/ neuron	Total length of minor processes/neuron
Polylysine	295±18	225±15	65±4
Laminin (10 μg/ml)	530±18*	450±20*	70±8
Laminin (20 μg/ml)	680±25*	560±18*	75±5

n = 300 cells per experimental condition; data are expressed as the mean ± s.e.m.

Length values are expressed in μm.

*Values significantly different from those of cells growing on polylysine alone.

Table 2. Effect of laminin on neuronal shape parameters by cerebellar macroneurons in 48 hour cultures

Group	Total neuritic no./neuron	No. of axons/ neuron	No. of axonal branches	No. of minor processes/ neuron
Polylysine	5±0.15	1.05±0.03	1.50±0.3	4.10±0.25
Laminin (10 μg/ml)	10±0.50*	1.35±0.15	4.25±0.5*	4.35±0.30
Laminin (20 μg/ml)	14±0.45*	1.30±0.20	7.50±0.7*	4.60±0.60

n = 300 cells per experimental condition; data are expressed as the mean ± s.e.m.

Length values are expressed in μm.

*Values significantly different from those of cells growing on polylysine alone.

Table 3. Changes in total neuritic and axonal length after the acute addition of laminin to cerebellar macroneurons growing on polylysine

		Hours after the addition of laminin to the culture medium			
Treatment	Measurement	0	6	12	24
Non-treated					
	Total neuritic length	158±12	185±14	230±12	295±10
	Total axonal length	110±10	150±12	195±14	245±20
Laminin (20 μg/ml)					
	Total neuritic length	158±10	228±12	312±15*	436±20*
	Total axonal length	116±10	195±10*	258±16*	360±22*

All experiments started 24 hours after plating.

Length values are expressed in μm. Data represent the mean ± s.e.m. A total of 100 cells was analyzed per time point per experimental condition.

*Values significantly different from those of non-treated cells.

mAb 150, but stain intensely with the antibody 531; this labeling is completely lost when cells are extracted with detergents before fixation. By contrast, in neurons growing on polylysine, antibody 531 not only stains intensely minor processes but also axons, while a moderate but selective labeling of the distal end of axonal processes is observed with the mAb 150. Quantitative fluorescence measurements confirmed these observations and revealed unambiguously that a significant increase in mAb 150 immunolabeling closely parallels axonal elongation in neurons growing on a laminin-containing substrate (Fig. 1c).

Dissociated cerebellar macroneurons cultured either on polylysine or laminin express tau proteins during their in vitro development (Figs 1d and 2b; see also Caceres and Kosik, 1990; Caceres et al., 1992; Ferreira et al., 1989). The present observations show that laminin also induces a delayed enhancement of tau protein expression; thus, after 2 days in culture we detected a significant increase in tau protein levels with respect to the values observed in control neurons (Fig. 1d). On the other hand, no differences in the induction profiles of MAP-1a or MAP-2 are observed between neurons growing on a laminin-containing substrate or polylysine alone (not shown).

MAP-1b and tau protein function during laminin-enhanced axonal growth

The high correlation between the dramatic incorporation of MAP-1b into microtubules and the enhancement of axonal elongation observed in the presence of laminin suggested a causal relationship between these two events. Therefore to test this possibility directly, we analyzed the characteristics of neurite outgrowth on laminin in cerebellar macroneurons treated with antisense oligonucleotides specific for the MAP-1b mRNA.

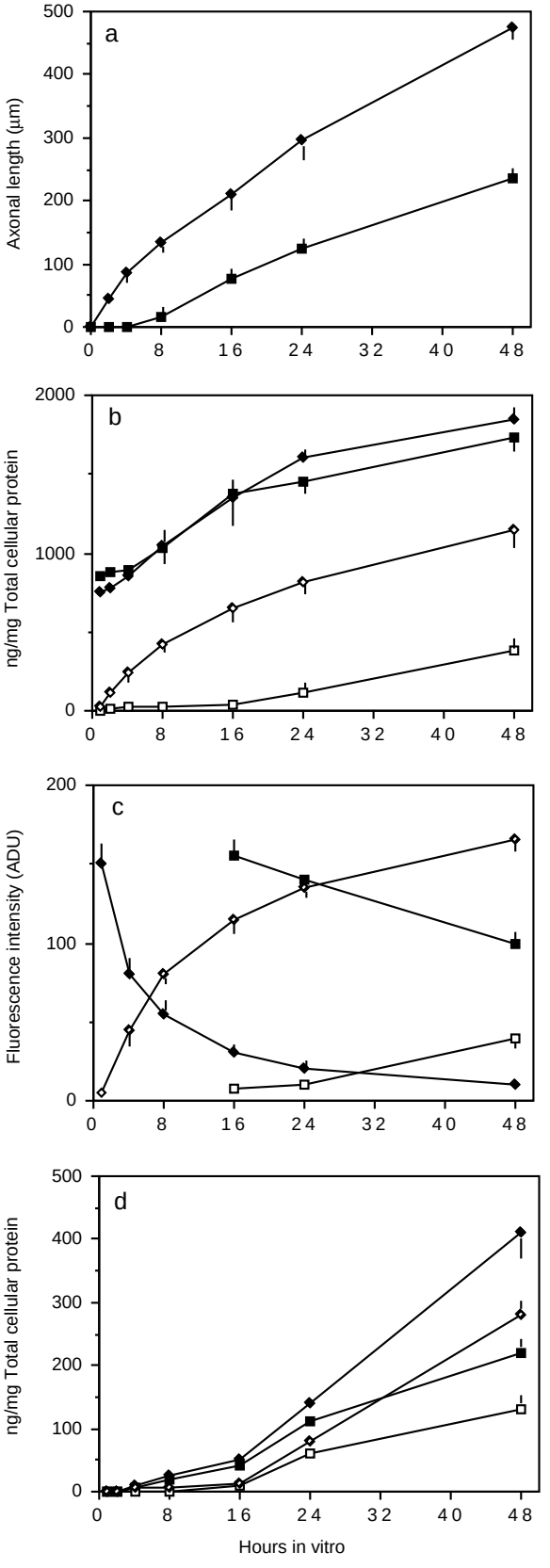


Fig. 1. (a) Graph showing the time course and extent of axonal elongation in cerebellar macroneurons cultured on polylysine (solid diamonds) or polylysine + laminin (10 μg/ml; solid squares). (b) The pattern of expression and incorporation into the cytoskeleton of MAP-1b in cultured cerebellar macroneurons as determined by dot immunobinding assays of whole cell homogenates or cytoskeletal fractions with the mAb AA6. The solid symbols represent total (T.) MAP-1b protein levels in neurons growing on polylysine (squares, P.) or on a laminin-containing substrate (diamonds, L.). The open symbols represent the fraction of MAP-1b associated with the cytoskeleton (Ck.) in neurons growing on polylysine (squares, P.) or on a laminin-containing substrate (diamonds, L.). Each value represents the mean ± s.e.m. (c) Average intensity measurements of MAP-1b immunofluorescence within the distal third of growing axons of cerebellar macroneurons cultured on polylysine (squares, P.) or on a laminin-containing substrate (diamonds, L.) and stained with the rabbit antiserum 531 (Ab 531) or the mAb 150 (Ab 150). Each value represents the mean ± s.e.m. Note that in neurons growing on laminin the increase in mAb 150 immunofluorescence closely parallels axonal elongation and the decrease in Ab. 531 immunolabeling. A total of 30 cells was analyzed for each time point and experimental condition. (d) The pattern of expression and incorporation into the cytoskeleton of tau proteins in cultured cerebellar macroneurons as determined by dot immunobinding assays of whole cell homogenates or cytoskeletal fractions with the mAb tau-1. The solid symbols represent total (T.) tau protein levels in neurons growing on polylysine (squares, P.) or on a laminin-containing substrate (diamonds, L.). The open symbols represent the fraction of tau associated with the cytoskeleton (Ck.) in neurons growing on polylysine (squares, P.) or on a laminin-containing substrate (diamonds, L). Each value represents the mean ± s.e.m.

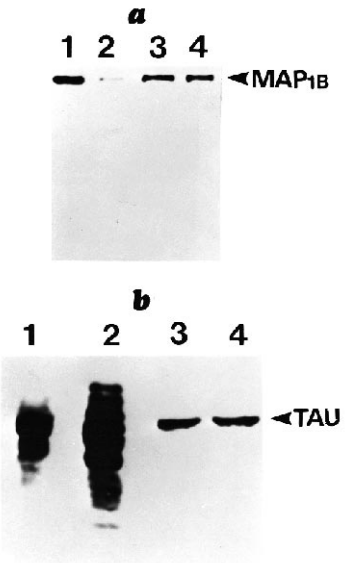


Fig. 2. (a) Western blot analysis with a mAb (clone AA6) specific for MAP-1b revealed that cerebellar macroneurons express this protein at the time of plating. Lane 1, MAP-1b immunoreactive polypeptide from a 10-day-old rat brain microtubule preparation; lane 2, MAP-1b immunoreactive polypeptide from adult rat brain microtubules. Each lane was loaded with 5 μg of microtubular protein. The MAP-1b immunoreactive polypeptide is also present in whole cell homogenates prepared from cerebellar macroneurons cultured on polylysine (lane 3) or laminin (lane 4) for 2 hours. Lanes 3 and 4 were loaded with 30 μg of total cellular protein. (b) Western blot analysis of whole cell homogenates with the mAb tau-1 revealed that cerebellar macroneurons express tau protein. In these extracts the tau-1 antibody reacts with a polypeptide that migrates in a similar position to that of the slowest migrating tau protein from 10-day-old rat brain microtubules. Lane 1, Tau-1 immunoreactive polypeptides from a 10-day-old rat brain microtubule preparation; lane 2, Tau-1 immunoreactive polypeptides from adult rat brain microtubules. A tau-1 immunoreactive polypeptide is also present in whole cell homogenates prepared from cerebellar macroneurons cultured on polylysine (lane 3) or laminin (lane 4) for 24 hours (see also Ferreira and Caceres, 1989). Lanes 3 and 4 were loaded with 30 μg of total cellular protein.

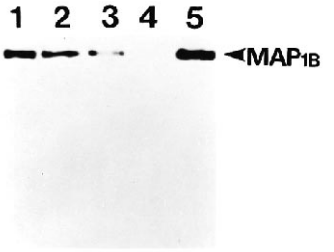


Fig. 3. Effect of the antisense oligonucleotide As1 on MAP-1b protein levels as revealed by western blot analysis of whole cell homogenates obtained from cerebellar macroneurons cultured for different time periods on a laminin-containing substrate and reacted with the mAb AA6. Lane 1, 4 hours after plating; lane 2, 8 hours after plating; lane 3, 16 hours after plating; lane 4, 24 hours after plating. Note the progressive decrease in MAP-1 b protein levels by contrast, no decrease in MAP-1b protein levels are detected in whole cell homogenates from 1-day-old cultures treated with the corresponding sense oligonucleotide and reacted with the mAb AA6 (lane 5); 40 μg of total cellular protein were loaded in each lane. Quantitative immunoblot analysis of whole cell homogenates from 1-day-old cultures reacted with the mAb AA6 and iodinated Protein A (see Materials and Methods) also revealed the dramatic decrease in MAP-1b protein levels (sense-treated: 1.2 ± 0.12 μg of MAP-1b/mg of total cellular protein; As1-treated cells: less than 50 ng of MAP-1b/mg of total cellular protein).

The addition of the MAP-1b antisense oligonucleotide As1 at a dose of 50 μM, but not of the corresponding sense-strand oligonucleotide, to cells growing on polylysine or laminin produces a significant reduction in MAP-1b protein levels as determined by western blot analysis (Fig. 3) or dot immunobinding assay (Fig. 4a) of whole cell homogenates reacted with the mAb AA6. Inhibition of MAP-1b expression becomes evident after 16 hours of antisense treatment and by 1 day only trace amounts of protein are detected in the cell homogenates (Figs 3 and 4a). The levels of total tubulin and of several MAPs, including MAP-1a, MAP-2 or tau, are not modified by treatment of cerebellar macroneurons with the antisense oligonucleotide AS1 (Fig. 4b).

MAP-1b suppression has different effects on neurite extension depending on whether the neurons grow on polylysine or on a laminin-containing substrate. On polylysine, no differences between the MAP-1b antisense-treated neurons and the control ones are detected in the time-course of neurite extension, the number of neurites, and the length of minor neurites or of the axonal plexus (Fig. 4c). Similarly, MAP-1b antisense oligonucleotides do not prevent neurite formation in neurons cultured in the presence of laminin. However, As1 at doses of 50 or 25 μM selectively and significantly reduces the enhancement in the rate and extent of axonal elongation induced by laminin (Fig. 4d). This inhibition becomes evident after 16 hours in culture, which is highly coincident with the reduction in MAP-1b protein levels, and persists as long as the cells are maintained in medium containing antisense oligonucleotides. A selective reduction in the length of the axonal plexus is also observed when the cells are treated with equivalent doses of the MAP-1b antisense oligonucleotides As2 or As3 (Table 4). When the medium is replaced with fresh medium lacking antisense oligonucleotides, the cells re-express MAP-1b and increase the rate of axonal elongation (Table 5).

A similar analysis performed with the tau antisense oligonucleotide RT 11/14 (11-13) revealed that while tau suppression completely inhibits axonal formation in neurons growing on polylysine (Fig. 4e), it has little effect in the presence of laminin (Fig. 4f). These neurons are capable of extending axon-like neurites with a time course identical to that of sense-treated or non-treated cells. It is only after 48 hours in the presence of RT11/14 that we detected a slight but significant reduction of axonal length (Fig. 4f).

Laminin also causes axonal formation in tau-suppressed neurons when it is acutely added to the tissue culture medium. For this experiment, neurons growing on polylysine were treated with RT 11/14 (50 μM) for 2 days starting 1 hours after plating. At the end of the first day in vitro, when 90% of the cells have failed to elaborate an axon and display a significant and selective reduction of stable microtubules (Caceres and Kosik, 1990), a single dose of laminin (20 μg/ml) was added to the culture medium; cells were then analyzed several hours

later. Under this condition more than 90% of tau-suppressed neurons are capable of extending an axon-like neurite (Fig. 5). This phenomenon is paralleled by the appearance of stable polymer within the axonal shaft and the incorporation of phosphorylated MAP-1b into axonal microtubules (Fig. 5). Quantitative fluorescence measurements revealed that laminin-induced axonal formation in tau suppressed neurons is not accompanied by increases in the MAP-1a or MAP-2 staining

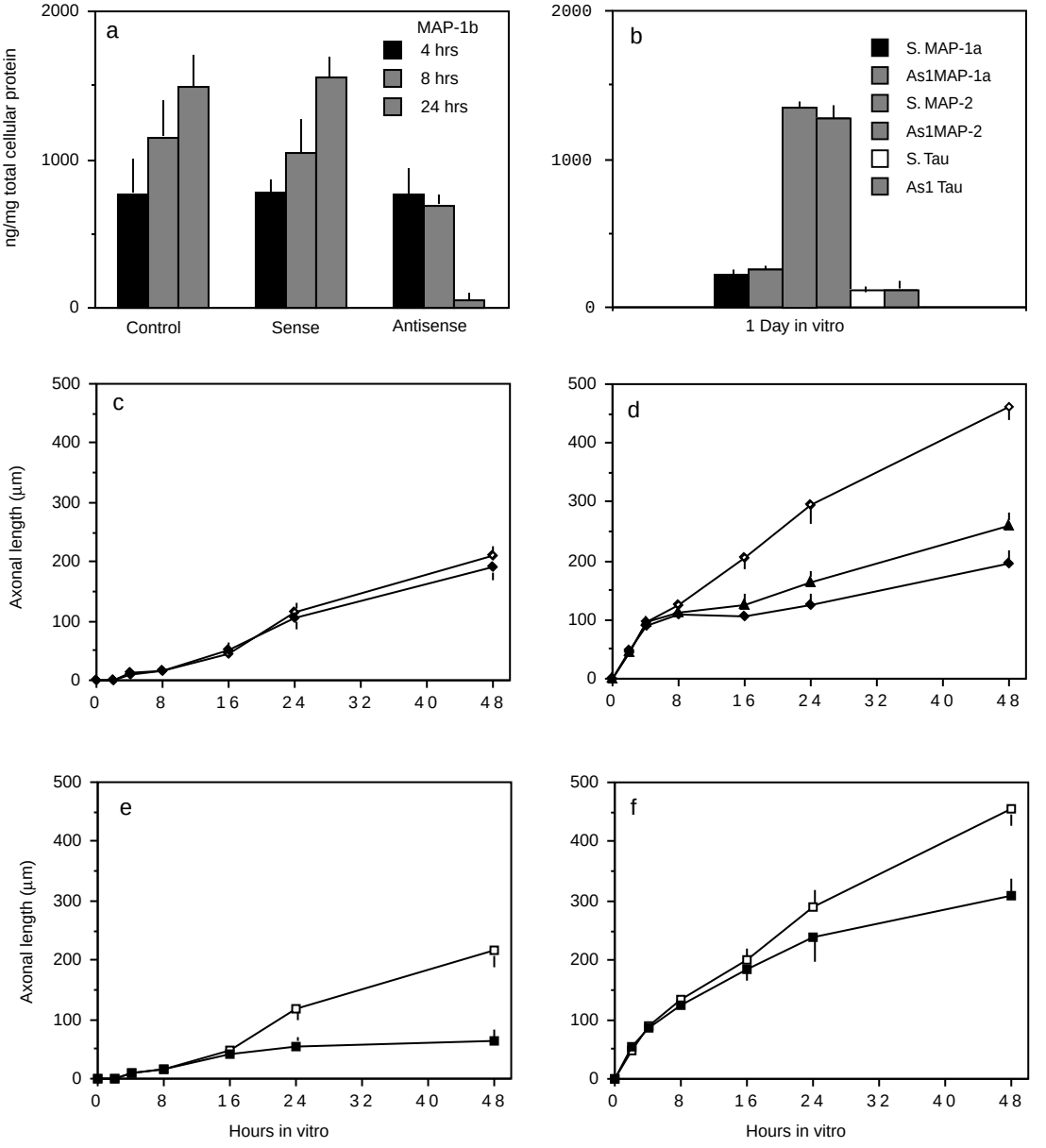


Fig. 4. (a) Effect of the antisense oligonucleotide As1 on MAP-1b protein levels as determined by dot immunobinding assays with the mAb AA6. Whole cell homogenates were prepared from cultured neurons 4, 8 and 24 hours after plating; note the dramatic decrease in MAP-1b protein levels in the antisense treated cultures 24 hours after plating. (b) Quantification of MAP-1a, MAP-2 and tau protein levels in whole cell homogenates prepared from 1 DIV (day in vitro) cultures as revealed by dot immunobinding assays with specific antibodies. MAP-1a protein levels in sense- (S. MAP-1a) and As1-treated (As1 MAP-1a) cultures; MAP-2 protein levels in sense- (S. MAP-2) and As1-treated (As1 MAP-2) cultures; tau protein levels in sense- (S. tau) and As1-treated (As1 tau) cultures. Note that there are no differences in MAPs protein levels between the sense- and antisense-treated cultures; the levels of these MAPs are almost identical to those detected in non-treated cultures. (c) Graph showing the time course and extent of axonal elongation in cultured cerebellar macroneurons growing on polylysine (P.) and treated with a sense MAP-1b oligonucleotide (open diamonds; S. MAP-1b) or 50 μM As1 (solid diamonds; AS. MAP-1b). (d) An equivalent graph to that shown in c but from neurons cultured on a laminin-containing substrate (L.); note that after 16 hours in culture there is a dramatic inhibition of axonal length in the antisense-treated cultures. (e) Graph showing the time course and extent of axonal elongation in cultured cerebellar macroneurons growing on polylysine (P.) and treated with a sense tau oligonucleotide (open squares; S. tau) or RT 11/14 (solid squares; AS. tau). (f) An equivalent graph to that shown in e but from neurons cultured on a laminin-containing substrate (L.); note that 48 hours after plating there is a significant reduction of axonal length in the RT11/14 treated cultures. Since in neurons growing on polylysine axons begin to develop after 20 hours in culture, the length of the longest of the minor neurites was used as an equivalent measurement at earlier time intervals. At least 100 cells were analyzed for each time point and experimental condition.

Table 4. Effect of MAP-1b antisense oligonucleotides on neurite outgrowth in cerebellar macroneurons after development in culture for 1 day

Treatment	Neurites per neuron	Axonal length (μm)	Total neuritic length (μm)
Cells growing on polylysine			
No-treatment	3.85±0.10	120±12	155±10
Sense-treated (50 μM)	3.90±0.20	125±10	160±15
Antisense-treated (As1, 50 μM)	3.60±0.12	118±14	150±12
Antisense-treated (As1, 25 μM)	3.80±0.14	124±12	155±10
Cells growing on laminin (10 μg/ml)			
No-treatment	7.20±0.48	215±20	260±22
Sense-treated (50 μM)	6.90±0.30	220±15	265±10
Antisense-treated (As1, 50 μM)	3.60±0.20*	145±15*	178±12*
Antisense-treated (As1, 25 μM)	3.90±0.20*	170±10*	205±12*
Antisense-treated (As2, 25 μM)	3.80±0.15*	165±10*	195±14*
Antisense-treated (As3, 25 μM)	4.20±0.20*	160±14*	200±12*

n = 200 cells per experimental condition; data are expressed as the mean ± s.e.m.

*Values significantly different from those of non-treated or sense-treated neurons.

Table 5. The inhibitory effects of As1 on MAP-1b protein levels and laminin-enhanced axonal extension are reversible

	Hours after the release from the antisense treatment		
Antisense treatment	0	12	24
Cells growing on polylysine			
Non-treated			
MAP-1b protein levels	1.60±0.40	1.66±0.28	1.70±0.30
Axonal length	120±15	180±10	235±15
As1-treated (50 μM)			
MAP-1b protein levels	0.05±0.05	0.0±0.12	0.90±0.20
Axonal length	125±12	165±14	215±20
Cells growing on laminin (10 μg/ml)			
Non-treated			
MAP-1b protein levels	1.65±0.29	1.68±0.30	1.75±0.20
Axonal length	260±20	350±28	480±30
As1-treated (50 μM)			
MAP-1b protein levels	0.05±0.02	0.40±0.18	0.95±0.15
Axonal length	125±12	230±10	350±22

Cultures were treated with the MAP-1b antisense oligonucleotide (As1) for 24 hours; after that time period the medium was replaced by fresh one lacking the As1 oligonucleotide and the cells analyzed at different time intervals after the release from the antisense treatment.

Data are expressed as the mean ± s.e.m.

MAP-1b protein levels were determined by dot immunobinding assays. Values are expressed in μg/mg of total cellular protein.

Length values are expressed in μm. A total of 100 cells was analyzed for each time point and experimental condition.

of the cell body and neurites of neurons fixed before or after detergent extraction performed under microtubule stabilizing conditions.

In a final set of experiments, we treated cerebellar macroneurons growing on a laminin-containing substrate with a combination of the As1 (25 μM) and RT11/14 (25 μM) oligonucleotides. Fig. 6a shows that after 16 hours in the presence of the mixture of antisense oligonucleotides there is a dramatic inhibition of axonal elongation; moreover, most of the cells retract their axons and by 30 hours after plating they loose their morphological polarity, displaying a symmetrical array of short neurites. Release of the cultured neurons from any one of the antisense oligonucleotides results in the regrowth of axons; a lag of 12-15 hours existed between the changing of the medium and the appearance of the axon (Fig. 6b). By contrast, no inhibition of axonal formation is detected in cells treated with As1 or RT 11/14 plus any one of the sense-strand oligonucleotides used in this study (Fig. 6c).

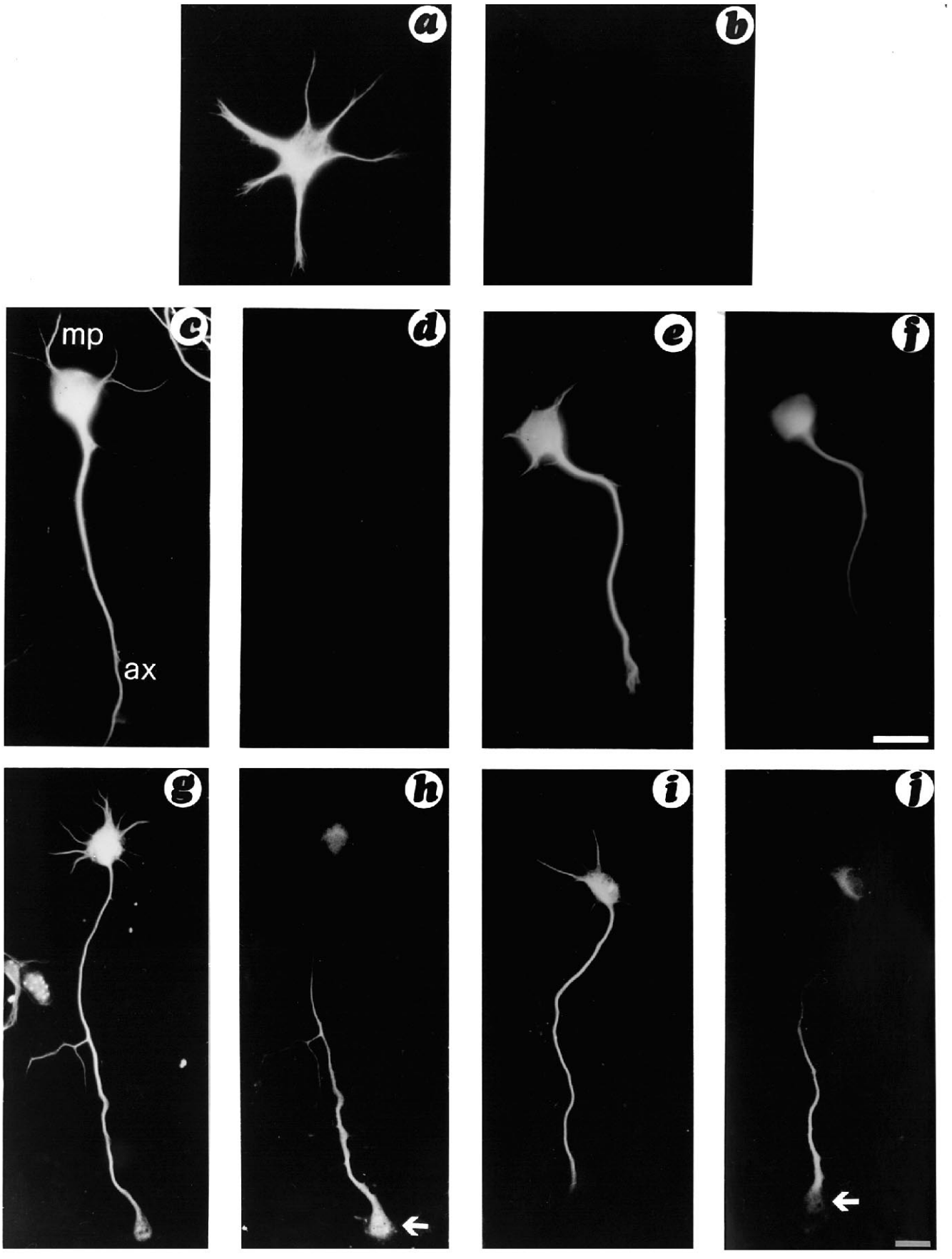
DISCUSSION

The present observations indicate that in cerebellar macroneurons laminin causes a rapid, dramatic and selective stimulation of axonal elongation. These observations confirm and extend previous studies in mesencephalic (Chamack and Prochiantz, 1988), sympathetic (Lein and Higgins, 1989) and hippocampal neurons (Lein et al., 1992; Lochter and Schachner, 1993) showing that laminin, as well as other ECM molecules, are capable of stimulating the onset of neuronal polarity. More importantly, in this study we show for the first time that laminin affects process extension by modulating the activity and pattern of expression of two well characterized MAPs, namely MAP-1b and tau.

Laminin promotes MAP-1b participation in axonal elongation

MAP-1b is a developmentally regulated microtubule protein that is specially prominent in neurons that are actively extending axons (Calvert and Anderton, 1985; Calvert et al., 1987; Fischer and Romano-Clarke, 1991; Riederer et al., 1986). Immunofluorescence studies have demonstrated that MAP-1b is highly enriched at the distal end of growing axons (Avila et al., 1994; Black et al., 1994; Fischer and Romano-Clarke, 1991; this study). An increase in MAP-1b phosphorylation occurs during brain development (Avila et al., 1994; Calvert et al., 1987; Sato-Yoshitake et al., 1989) and neurite extension in PC12 cells (Aletta et al., 1988; Brugg and Matus, 1988; Greene et al., 1983); and cultured hippocampal pyramidal neurons (Boyne et al., 1995); besides suppression of MAP-1b by antisense oligonucleotides in PC12 cells reduces NGF-induced neurite outgrowth (Brugg et al., 1993). These observations raised the idea of MAP-1b participating in axonal growth and that this function is modulated by phosphorylation. Our results are fully consistent with this proposal and provide direct evidence suggesting an important role for MAP-1b in axonal elongation.

The data presented here suggest that laminin can promote the in vivo usage of MAP-1b for axonal formation. In cerebellar macroneurons growing in the presence of laminin, MAP-1b is incorporated into microtubules, and as shown by the antisense experiments, becomes functionally involved in axonal elongation. Several lines of evidence suggest that the incorporation of MAP-1b into microtubules is a prerequisite for its participation in axonal formation. For example, in neurons growing on polylysine, where the protein is not associated with microtubules, there is no correlation between the time course of neurite extension and the high levels of MAP-1b expressed by the neurons. In contrast, in the presence of laminin, MAP-1b incorporation into microtubules closely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parallels axonal outgrowth and elongation. Perhaps more significantly, suppression of MAP-1b by antisense oligonucleotides is only effective in reducing axonal extension in neurons growing in the presence of laminin, but not on polylysine.

The enhanced incorporation of MAP-1b into microtubules is not related to an overall increase in the expression of microtubular proteins, since no differences are detected in the induction profiles of MAP-1a, MAP-1b, or MAP-2 between neurons growing on a laminin-containing substrate or polylysine alone. It may be argued that any substance capable of enhancing neurite extension and/or axonal elongation may increase the incorporation of MAP-1b into microtubules. However, estradiol 17-β, a substance capable of selectively stimulating axonal extension in cultured hippocampal (Blanco et al., 1991) and hypothalamic neurons (Diaz et al., 1992; Ferreira and Caceres, 1991) has no effect on the levels of MAP-1b associated with the cytoskeleton, even though it significantly stimulates tau protein expression and its incorporation into microtubules (Ferreira and Caceres, 1991; A. Cáceres, unpublished observations).

In the particular case of laminin, our results suggest that this molecule may initially stimulate axonal elongation by regulating the post-translational processing of MAP-1b. Thus, in the presence of laminin we detected a significant increase in phosphorylated MAP-1b that is likely to increase its ability to interact with microtubules, and hence regulate their dynamic properties. This is dramatically illustrated in tau-suppressed neurons growing on polylysine where the addition of laminin to the culture medium induces axonal formation, microtubule stabilization, and a significant incorporation of phosphorylated MAP-1b into microtubules. Taken collectively these observations support recent proposals suggesting that environmental signals, including ECM molecules, modify cell shape by modulating the activity of kinases or phosphatases that regulate the phosphorylation status of cytoskeletal elements (see Avila et al., 1994; Sastry and Horwitz, 1994).

Fig. 5. (a,b) Inhibition of axonal formation in tau antisense-treated neurons growing on polylysine. The micrographs show an example of a neuron that was treated with RT11/14 (50 μM) starting 1 hour after plating, fixed 30 hours later, and double labeled with a rabbit antiserum against total tubulin (a) and the mAb tau-1(b). Note that the cell is arrested at stage II of neuritic development and lacks tau immunolabeling. (c-f) The addition of laminin (20 μg/ml) to the culture medium of tau antisense-treated neurons growing on polylysine induces the elaboration of an axon-like neurite. The double immunofluorescence micrographs show examples of cells treated with RT11/14 (50 μM) starting 1 hour after plating, fixed 30 hours later, and that received a single dose of laminin 8 hours before fixation. The cells were double labeled with a rabbit antiserum against total tubulin (c,e,g,i) and with mAbs against tau (Clone tau-1, d), acetylated α-tubulin (Clone 6-11B-1, f), and phosphorylated MAP-1b (Clone 150, h, j). Note that all neurons have extended a long axon-like neurite (ax) that significantly exceeds in length the remaining minor processes (mp). This phenomenon is accompanied by the appearance of acetylated microtubules within the axonal shaft (f) and phosphorylated MAP-1b (h,j) within the distal third of the growing axons, including their growth cones (arrows). Note that the labeling generated by the mAb 150 is present in axons independent of whether the cells are fixed prior (h) or after (j) detergent extraction under microtubule stabilizing conditions. Bars: (a-f), 10 μm; (g-j), 10 μm.

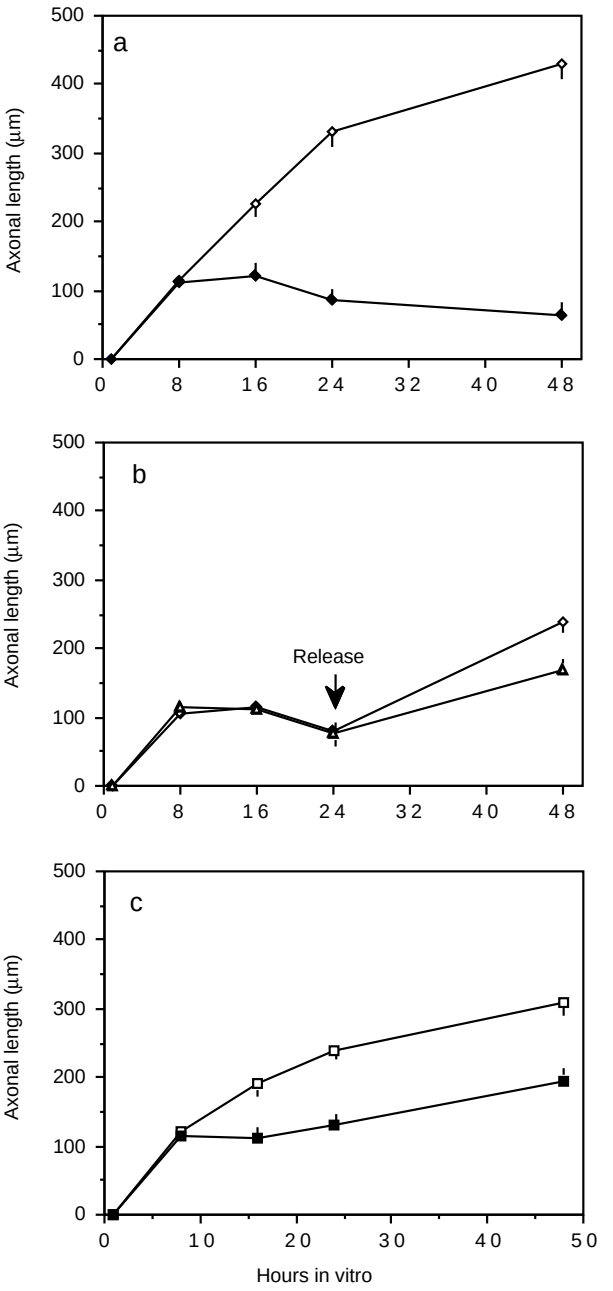

Fig. 6. (a) Graph showing the time course and extent of axonal elongation in cultured cerebellar macroneurons growing on a laminin-containing substrate and treated with a combination of MAP-1b and tau sense oligonucleotides (open diamonds) or As1+RT11/14 (solid diamonds). Note the dramatic reduction of axonal length observed in the double antisense-treated neurons. (b) Graph showing that release (Re.) of double suppressed (Ds.) neurons from As1 (open diamonds; As1 Re.) or RT 11/14 (open triangles; RT11/14 Re.) results in an increase in axonal length. Release from any one of the antisense oligonucleotides was performed 24 hours after plating (arrow). (c) Graph showing the rate and extent of axonal elongation in neurons treated with a MAP-1b sense oligonucleotide plus RT11/14 (open squares) or a sense tau oligonucleotide plus As1 (solid squares). Note that none of these treatments prevents axonal formation, even though there is a significant reduction of axonal length when compared with sense treated or non-treated cultures. Antisense oligonucleotides were used at 25 μM each, while a 50 μM dose was employed in the case of each of the sense oligonucleotides.

Laminin reveals the existence of MAP-1b/tau functional redundancy

In previous studies we have shown that tau antisense-treated cerebellar macroneurons failed to extend axon-like neurites (Caceres and Kosik, 1990; Caceres et al., 1991, 1992). Inhibition of neurite elongation was also observed in PC12 cells treated with a different set of antisense oligonucleotides (Hanemaaijer and Ginzburg, 1991) or after transfection with an antisense cDNA (Esmaeli-Azad et al., 1994). On the other hand, PC12 cells overexpressing tau extend neurites more rapidly than control ones (Esmaeli-Azad et al., 1994), and non-neuronal cells infected with a baculovirus containing a tau cDNA insert extend long axonal processes, in which microtubule bundles resembling those of axons are formed (Chen et al., 1992; Knops et al., 1991). It was on the basis of these observations that tau was thought to be crucial for axonal elongation. However, a recent study demonstrated that axon formation is not significantly altered in tau-deficient mice (Harada et al., 1994), and therefore tau was not considered essential in neurodevelopment. The work here suggests the existence of a compensatory mechanism whereby MAPs subserve redundant functions.

One explanation for the phenotype induced by the antisense oligonucleotide suppression of tau in cerebellar macroneurons was that non-specific inhibitory actions are responsible for the blockade of axonal formation (Caceres and Kosik, 1990; Caceres et al., 1991, 1992). This explanation is unlikely because, as shown here, tau antisense oligonucleotides do not always block axonal formation. According to the results here, inhibition of axonal extension in tau-suppressed neurons may not only result from the absence of tau, but also from the lack of compensatory mechanisms. In this case, MAP-1b seems not to be capable of replacing tau protein function when cerebellar macroneurons are grown on polylysine.

On the other hand, in the presence of laminin, neurons seem to use cooperatively or alternatively MAP-1b and tau for sustaining axonal elongation. Two aspects should be highlighted concerning this proposal. First, in neurons growing on laminin neither the single suppression of tau, nor of MAP-1b blocks axonal formation, even though the rate of elongation is significantly reduced. Secondly, the co-expression of MAP-1b and tau, as well as their incorporation into microtubules closely paralleled the enhanced rate of axonal growth displayed by neurons that develop on laminin. Conversely, the suppression of both of these MAPs completely prevents axonal extension; however, and perhaps more importantly, release of the neurons growing on laminin from any one of the antisense oligonucleotides results in axonal formation. The concomitant artificial condition of no laminin allows the tau-dependent phenotype to become manifest. Functional redundancy among cytoskeletal elements seems not to be an unusual phenomenon (Brown, 1993; Takeuchi et al., 1994) in the particular case of MAPs; it is interesting to note that in a PC12 clone displaying significant increases in neurite length and stability high molecular weight tau proteins, rather than the low molecular weight species, may modulate neuronal morphology (Teng et al., 1993). Interactions among MAP family members, extracellular molecules and environmental signals participate in the regulation of neuronal morphogenesis.

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