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Identification of a neurite outgrowth-promoting domain of laminin using synthetic peptides

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We have identified a synthetic peptide derived from the B2-chain of mouse laminin, Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile (p20), which simulates the neurite outgrowth-promoting activity of the native molecule. In organotypic cultures, neurons from newborn mouse brain or embryonic peripheral nervous system responded by extensive neurite outgrowth for native laminin or the peptide p20 in the culture medium. If rat cerebellar neurons were grown on laminin, 1–5 μM (1–5 μg/ml) of peptide p20 in the culture medium competed with laminin and inhibited neuronal attachment and neurite outgrowth, whereas higher concentrations (> 50 μM; > 50 μg/ml) had a specific neurotoxic effect. When peptide p20 was used as the culture substratum, neurite outgrowth in cerebellar cultures was up to 60% of that seen on native laminin. Our results indicate that a neurite outgrowth-promoting domain of laminin is located in the α-helical region of the B2-chain, and is active for both central and peripheral neurons.

Laminin; Neurite outgrowth; Synthetic peptide

1. INTRODUCTION

Laminin is a 1000 kDa extracellular matrix protein and a basement membrane component [1,2] that has a multidomain structure with various functions [3–5]. Recent evidence suggests that apart from its structural role in basement membranes, laminin or laminin-like molecules [6,7] may play a role in brain development [8–11] and nerve regeneration [12–15]. Studies using monoclonal antibodies to proteolytic fragments of laminin have suggested that the neurite outgrowth-promoting domain of laminin may be near or in the heparin-binding region of the molecule [16–18]. Proteolytic fragments isolated from this area have further been shown to promote attachment of chicken peripheral neurons [16,18]. Since the general cell-attachment domain of laminin has been mapped to the P1-fragment of the molecule [19] and has recently been shown to lie in the B1-chain of the protein [20], it is possible that laminin has multiple sites for cell attachment as well as for neurite outgrowth. This view is supported by the presence of more than one receptor molecule for laminin [21–26].

In this paper, we identify a region in the B2-chain of mouse laminin, Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile, that has characteristics for a putative neurite outgrowth-promoting domain of laminin. This can be concluded since nanomolar concentrations of synthetic peptides derived from this region simulate the neurite outgrowth function of native laminin.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis and coupling to carrier proteins*

Peptides were synthesized by the solid-phase method [27] us-

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ing a Vega Copler (250C) peptide synthesizer and *t*-Boc chemistry. The additional Gly-Cys in the carboxy-terminal was added to facilitate coupling of the peptides to carrier proteins. The sequences of the purified peptides were confirmed with gas-phase sequencer (model 470 A, Applied Biosystems). Peptide p20, RNIAEIIKDI (GC), and peptide p31, CDPGYIGSR(GC), were coupled to bovine serum albumin (BSA, Sigma, St. Louis, MO) via the carboxy-terminal cysteine as described [28]. Using this procedure approx. 20 peptide molecules were attached to one albumin molecule. The concentrations of albumin coupled peptides were always indicated according to the concentration of the peptide. The BSA-coupled peptides were used in cell-attachment studies only. In all other experiments uncoupled free peptides were applied.

2.2. *Organotypic cultures*

In Trowell-type organotypic cultures, newborn mouse spinal cord and cerebellum or 16-day-old mouse embryo dorsal root ganglia (DRG) were grown on Nuclepore filters (pore size 1.0 μm; Pleasanton, CA) placed on a metal grid in a serum free, chemically defined NI-medium [29] with or without one of the following peptides in the culture medium: p20, p31, p32, P6 or P7 (see table 1). All peptides were used at 100 nM (0.1 μg/ml). In DRG cultures the medium also contained 10 nM of 7S-NGF (Collaborative Research, Lexington, MA). In some experiments, the cultures were grown in the presence of soluble native mouse laminin, also applied at 100 nM (100 μg/ml). After 24–48 h the cultures were fixed with cold (−20°C) methanol for 30 min, the tissue explant was scraped off and the filters were exposed to rabbit polyclonal antibodies to neurofilament proteins [30] for 24 h at 4°C as described [31].

2.3. *Survival and competition assays*

Glass coverslips were covalently coupled [32] with 100 μg/ml of purified mouse laminin [2]. The free peptides were usually added to the culture medium simultaneously with the cells that were mechanically dissociated from newborn rat (Wistar) cerebellum [31]. In survival studies the peptides were applied at 100 μg/ml and in competition assays at concentrations of 1–5 μg/ml. In time-lapse-video recording experiments, 50 μg/ml of peptide p20 was added to neurons that had already extended neurites on laminin. Time-lapse-video recordings were performed using a National NV-8030 time-lapse recorder installed with an Olympus IMT inverted research microscope with high magnification oil-immersion optics.

2.4. *Cell attachment and neurite outgrowth assays*

Glass coverslips were covalently coupled with 100 μg/ml (100–200 nM) of the peptides p20-BSA and p31-BSA alone or in combinations indicated. Cells from newborn rat (Wistar) cerebellum were mechanically dissociated [31], and plated on the coverslips at a density of 1×10^5/ml in the NI-medium [29]. Quantitation of attached neurons and their neurite extension was performed after 24 h by counting all live attached neurons with long neurites (>5 times cell soma) using a Leitz Labovert inverted microscope with phase-contrast optics. In these experiments, the entire 18 mm coverslip was screened, and all single live neurons counted. In each experiment 4–5 coverslips per each culture substrate were screened, and the arithmetical mean of attached neurons with neurites on laminin substrate in one experiment was considered to be 100% and the means on all the remaining substrates of that experiment compared with it. The total of six separate experiments (see above) formed the raw material of the figures.

3. RESULTS

3.1. *Neurite outgrowth-promoting activity of soluble laminin and peptide p20 in organotypic cultures*

Explants of newborn mouse spinal cord, cerebellum and embryonic DRG extended short neurites through the pores of the Nuclepore filter within 24 h (fig.1A,C,E). In the presence of 100 nM (0.1 μg/ml) of the free peptide p20 (see table 1) or its longer derivative p32 (see table 1), both spinal cord and cerebellar neurons showed extensive neurite outgrowth through the pores of the filter (fig.1B,D). Neurite outgrowth in the DRG cultures was also stimulated, but the response was less dramatic (fig.1F). Higher concentrations of peptide p20 (up to 100 μg/ml) did not improve the neurite-outgrowth response, nor do they inhibit it. The non-neuronal cell-attachment decapeptide of laminin (p31; see table 1) or other control peptides tested (P6, P7; see table 1) had no effect on neurite outgrowth in this culture system. If 100 nM (100 μg/ml) of native laminin was included in the culture medium, neurite outgrowth through the pores of the filter was comparable to that seen in the presence of peptide p20 (fig.1G).

3.2. *Neurite outgrowth of neurons on laminin in the presence of synthetic peptides in the culture medium*

3.2.1. Neuronal survival

If newborn rat cerebellar neurons or mouse embryo DRG were plated on laminin coated glass coverslips, long neurites extended within 12 h (fig.2a,c). Addition of 100 μM (100 μg/ml) of peptide p20 or its longer derivative p32, simultaneously with the cells, resulted in neuronal death within 12 h (fig.2b), and neurite outgrowth in the DRG cultures was inhibited (fig.2d). A time-lapse-video recording revealed that addition of 50 μg/ml of peptide p20 to the culture medium of neurons that had already extended neurites on laminin caused complete cessation of cytoplasmic streaming and movement of the growth cones and ruffling membranes within 2 h exposure to peptide p20 (not shown). The inhibitory effect of the peptide for

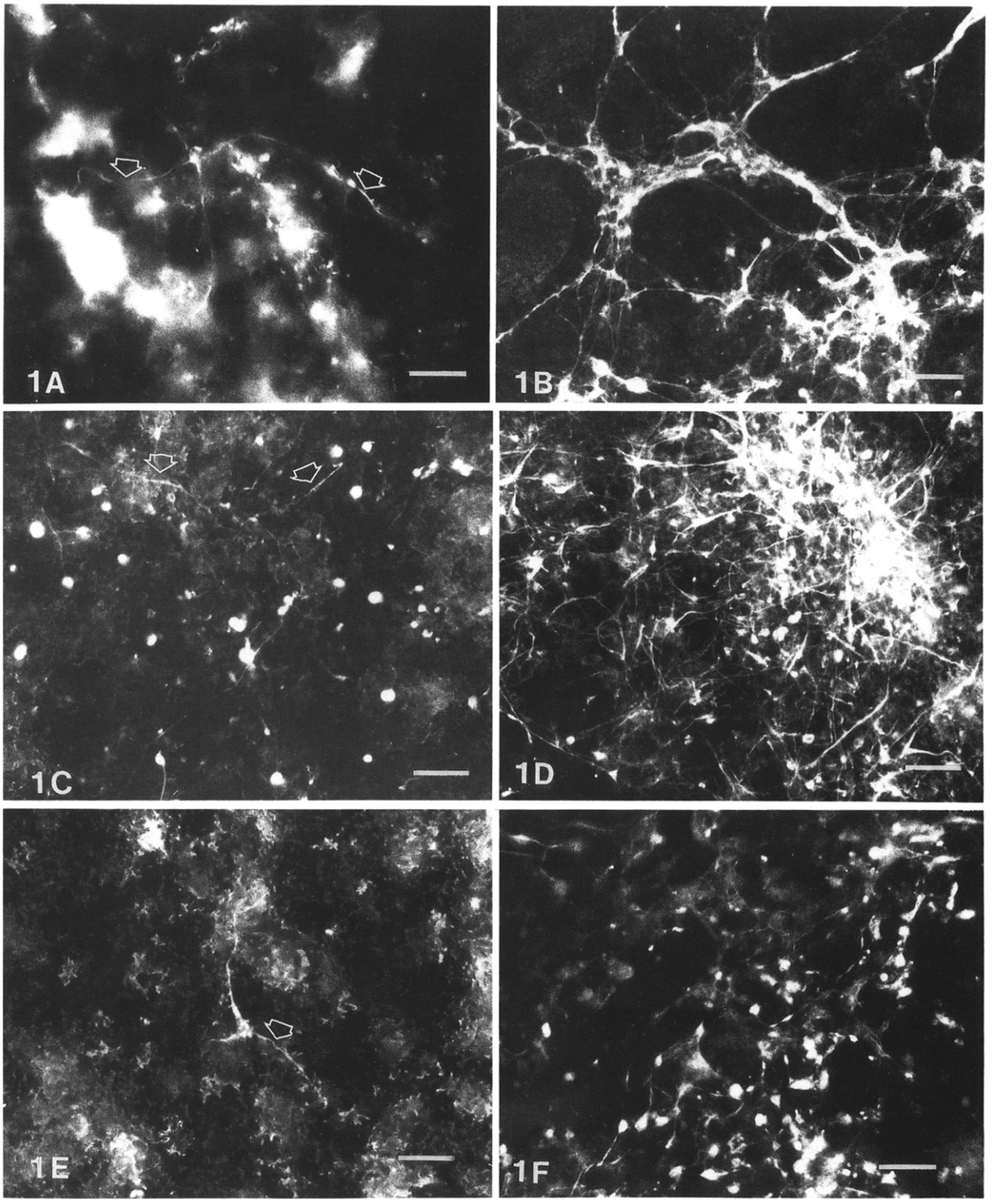
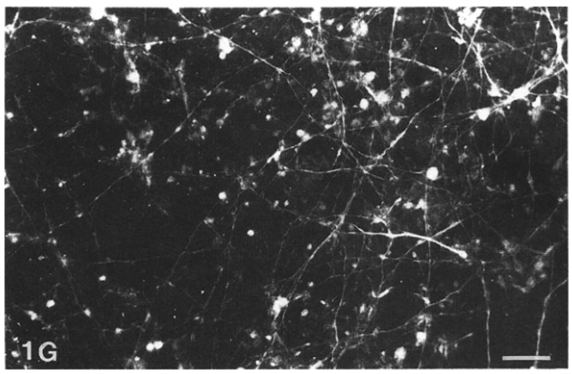


Fig.1. Demonstration of neurofilament protein immunoreactivity in organotypic cultures of newborn mouse spinal cord (A,B), cerebellum (C,D,G) and 16-day-old mouse embryo DRG (E,F). The fibres grown through the filter in 24 h are demonstrated in the absence (A,C,E) or in the presence (B,D,F) of 100 ng/ml (100 nM) of peptide p32 or laminin (G) in the culture medium. The presence of peptide p32 or laminin stimulated outgrowth of neurites in all areas (cf. A,C,E with B,D,F,G). In cultures of DRG (F) the effect of peptide p32 was less dramatic than with central neurons. Bar = 20 μm.



neuronal survival was dose dependent (fig.3) and specific for primary neurons. No toxicity was observed even with 200 μg/ml (0.2 mM) of peptide p20 for primary glial cells or for differentiation of 11-day-old mouse embryo kidney (not shown). The non-neuronal cell-attachment peptide p31 [20] or the other seven peptides tested (see table 1) did not influence neuronal survival on laminin when applied at similar concentrations (up to 200 μg/ml; table 1).

3.2.2. Competition assays

Rat cerebellar neurons were plated on laminin substrate and the free peptide p20 was simultaneously included into the culture medium at concentrations below the toxicity range (e.g., 1–5 μg/ml; 1–5 μM; see fig.3). Under these conditions, the attachment of neurons to laminin was reduced down to 55–70% of that seen on native laminin (fig.4), and outgrowth of long neurites was inhibited down to 20–40% of that seen on native laminin (fig.4).

3.3. *Attachment and neurite outgrowth of central neurons on synthetic peptides*

Rat cerebellar neurons were plated on glass coverslips coated with the peptides p20-BSA and/or p31-BSA at 100–200 nM (100–200 ng/ml), e.g. the equimolar concentration for 100–200 μg/ml (100–200 nM) of native laminin. BSA substratum was used as a control at a concentration of 600 ng/ml, which equals the BSA concentration of the peptide conjugates used at 200 ng/ml. The peptide p20-BSA (200 ng/ml) alone supported neuronal attachment nearly as well as the peptides p20-BSA (100 ng/ml) and

Table 1

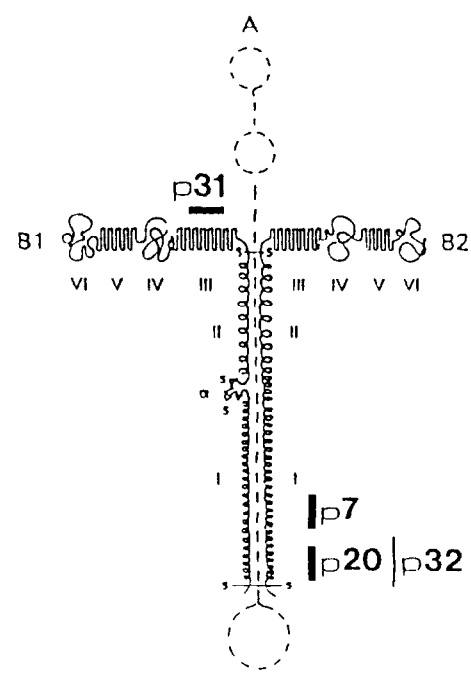
Synthetic peptides tested for inhibition of neurite outgrowth of rat central neurons on mouse laminin

200 μg/ml	Sequence [Ref.]	Inhibition activity
P1	DQQLLGGWGC [39]	–
P2	DEPEGIEEEGGERDDPSGC [39]	–
P3	SLIEESQNQQEKNEQELLEGC [39]	–
P4	ESKPEAEETC [40]	–
P5	GRGDSP [38]	–
P6	DYQKLNNAFGC (hydrophilic nonsense peptide)	–
P7	KAKDEMKASD[a] [33,34]	–
P20	RNIAEIIKDIGC[b] [33,34]	+ + +
P31	CDPGYIGSR [20]	–
P32	KQEAAIMDYNRNIAEIIKDIHNLED-IKKTL[b]	+ + +

[a] In our earlier work [41] this sequence was found to be neurotoxic at 100 μg/ml. Further analysis and improved peptide synthesis methods, however, showed that the peptide was inactive, and its neurotoxicity was due to impurities in the peptide preparation

[b] Four separate patches of peptide p20 were synthesized to confirm the reproducibility of its effects on neurons. Due to our earlier experience we lowered the concentrations of p20, and, thus, detected its non-toxic neurite outgrowth stimulating effects

The laminin peptides were derived from the cDNA sequence of the mouse B2-chain [33,34], and their relation to the domain map of laminin was presented as a diagram. It is notable that the sequence of the B2-chain of human laminin [36] is slightly different from that of the mouse in the area of peptide p20



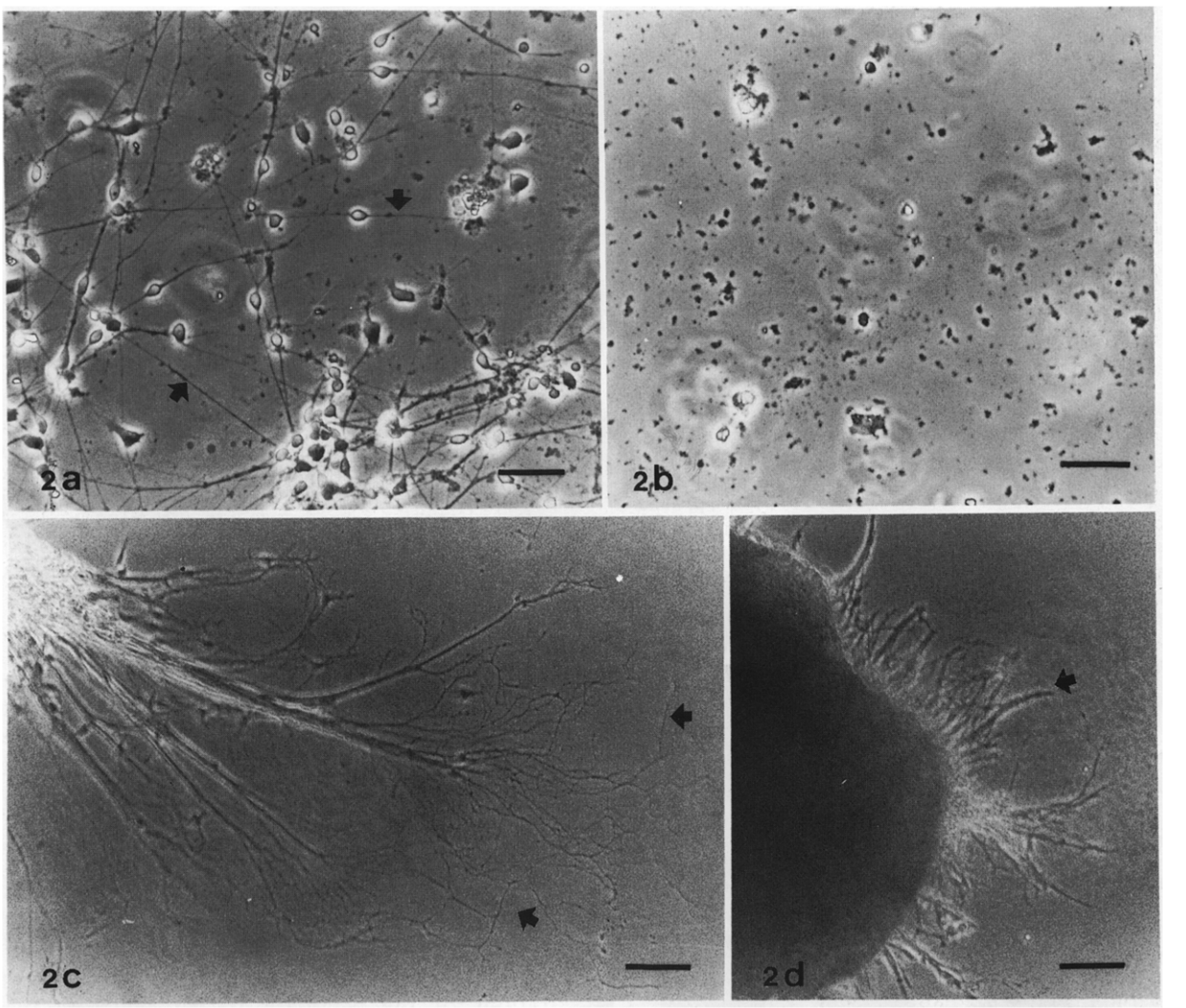


Fig.2. Phase-contrast micrographs of newborn rat cerebellar (a,b) and 16-day-old mouse embryo dorsal root ganglia (c,d) neurons grown on laminin in the absence (a,b) or in the presence (b,d) of 100 μg/ml (100 μM) of peptide p20. Neurons showed extensive neurite outgrowth on laminin (a,c, arrows). In the presence of 100 μg/ml of the peptide p20 (or p32) massive cell death occurred (b) and all neurite outgrowth was inhibited (d, arrow). Bar = 40 μm.

p31-BSA (100 ng/ml) used simultaneously as culture substratum (cf. p20 with p20 + p31 in fig.5). Outgrowth of neurites on p20-BSA alone (p20 in fig.5) was up to 60% of that seen on native laminin, and was comparable to the neurite outgrowth when the peptides p20-BSA and p31-BSA were used simultaneously (see p20-p31 in fig.5). Peptide p31-BSA (200 ng/ml; p31 in fig.5) alone supported attachment of neurons, but its effect on outgrowth of neurites was only marginally better than that of albumin (cf. BSA and p31 in fig.5).

4. DISCUSSION

We have identified a region in the B2-chain of mouse laminin, Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile (p20), which bears the criteria for a neurite outgrowth-promoting domain for primary central and peripheral neurons. This can be concluded,

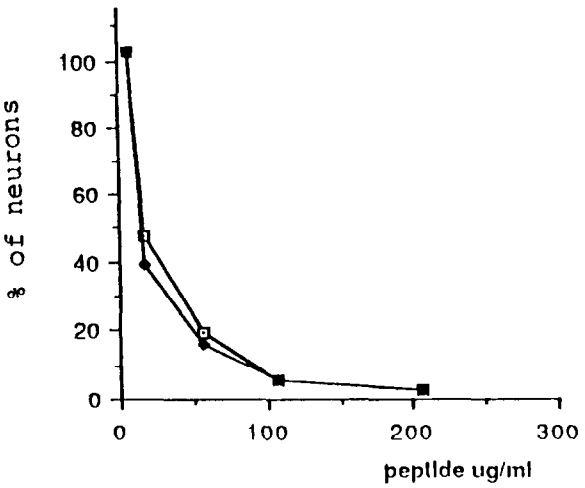


Fig.3. A dose-response curve for the effect of peptide p20 added to the culture medium on the number of neurons (■) and neurites (⊡) in cultures of newborn rat cerebellum grown on laminin. Concentrations higher than 50 μg/ml caused a specific neurotoxic effect, whereas concentrations of 1–5 μg/ml were non-toxic.

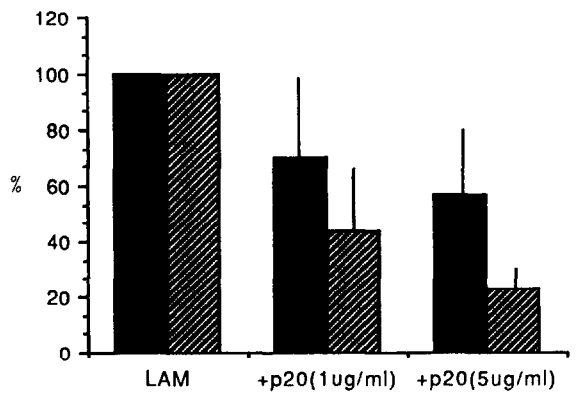


Fig.4. Attachment of neurons (■) and outgrowth of neurites (▩) in cultures of rat cerebellar neurons grown on laminin in the presence of 1–5 μM (1–5 μg/ml) of the free peptide p20 in the culture medium after 12 h in vitro. Both neuronal attachment and neurite outgrowth on laminin are inhibited. The most clearcut effect is the ability of peptide p20 (5 μg/ml) to inhibit outgrowth of neurites on the laminin substrate down to 30% of that seen on laminin.

since nanomolar (100–200 nM) concentrations of this peptide promote neurite outgrowth of central and peripheral neurons in organotypic cultures; compete with laminin for both neuronal attachment and neurite outgrowth; and simulate the effect of laminin when coupled to albumin and

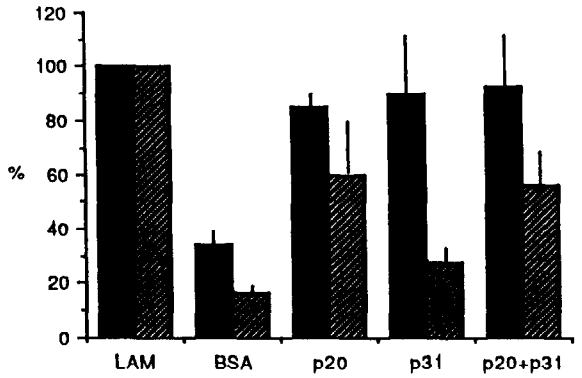


Fig.5. Neuronal attachment (■) and number of neurites (▩) in cultures of rat cerebellar neurons grown on LAM (100 μg/ml), BSA (0.6 μg/ml), p20-BSA (0.2 μg/ml), p31-BSA (0.2 μg/ml) or p20-BSA + p31-BSA (0.1 μg/ml each). The peptide p20-BSA alone was comparable in neuronal attachment and neurite outgrowth to the two peptides p20-BSA and p31-BSA together (p20 + 31). The peptide p31-BSA (p31) alone did not support neurite outgrowth any better than albumin, although it supported attachment of neurons.

included into the culture substratum. The decapeptide (p20) sequence was selected from the cDNA sequencing data available [33,34]. This sequence was chosen because it represented the amphiphilic peak [35] of the region of mouse laminin known to promote neurite outgrowth [16,18]. A closely related, but not identical sequence is present in the B2-chain of human laminin [36] and its effect in promoting neurite outgrowth of mouse or human neurons remains to be investigated.

We found that laminin and equimolar concentrations of the peptide p20, derived from the B2-chain of mouse laminin, support neurite outgrowth of central and peripheral neurons in organotypic cultures. This suggests that soluble laminin or peptide p20 may act as a stronger attractant for neurite outgrowth than the laminin present in the tissue explant itself. Since nanomolar concentrations of soluble laminin have been detected in the cerebrospinal fluid of adult mice [37], it is possible that laminin and/or soluble peptides, e.g. the neurite outgrowth-promoting peptide, may be released by the proteolytic machinery of the brain and exert functional or pathological response.

The ability of peptide p20 to simulate the effect of laminin at physiological concentrations and its specific neurotoxicity at high concentrations are of

particular interest, since they indicate that this sequence is specifically active for neurons. It is not possible to test, whether equimolar amounts of soluble laminin would be similarly neurotoxic, because this would require concentrations (0.1 g/ml; 100 mM) of laminin that would not remain in solution. Since insulin and many substances that have a biological function at physiological concentrations are toxic at high concentrations, a similar situation might be obtained in our experiments with peptide p20. Competition for neuronal attachment and neurite outgrowth with laminin suggests that the amino acid sequence of peptide p20 may be recognized by neuronal laminin receptors. Although this peptide sequence was chosen for its amphiphilicity and not for its hydrophilicity like the fibronectin [38] or laminin [20] cell-attachment peptides, the function of this region may be analogous to that of the fibronectin cell-attachment sequence, known to compete with fibronectin for fibroblast attachment and receptor binding [38]. The fact that the concentrations of the fibronectin tetrapeptide, effective in competition assays for fibroblast attachment [38], were higher than those of peptide p20, effective in our competition assay, may reflect the higher sensitivity of the non-dividing neurons as opposed to the fibroblasts.

Is the sequence of peptide p20 the only neuronal attachment and neurite outgrowth-promoting domain of laminin? If this were the case one would expect peptide p20 to substitute laminin completely. This is clearly not the case. As culture substratum, the peptide p20-BSA alone or together with the non-neuronal cell-attachment peptide (p31-BSA) simulated the effect of laminin, but could never fully substitute the native protein. Similarly, 10–50-fold molar excess of peptide p20 in the culture medium of neurons plated on laminin, did compete with the native protein, but inhibition of neurite outgrowth was not complete. Since laminin is such a large molecule its different chains may exert different effects on neurons, and interaction of several domains with each other or with other cell surface molecules may be needed for neuronal survival and maximal neurite outgrowth. This view is also supported by earlier work that has provided evidence for the role of the A-chain of laminin in promotion of neurite outgrowth [16], and by recent results that further imply that a 25 kDa proteolytic fragment of laminin, bearing both the region identified in this paper and the area reported by Engvall et al. [17], is capable of supporting attachment and neurite outgrowth of chicken peripheral neurons [18].

In previous studies, the non-neuronal cell-attachment domain of laminin has been localized in the P1-fragment of the molecule [19]. Using synthetic peptides Graf et al. [20] have recently demonstrated that the function of this domain could be substituted by a synthetic peptide from the B1-chain of laminin included within the P1-fragment. These authors further reported that their peptide sequence supported neuronal attachment, but no neurite outgrowth. Our results corroborate this work, since physiological concentrations of the peptide p31-BSA alone supported attachment of neurons, but did not promote outgrowth of neurites any better than BSA. Since the peptides p20-BSA and p31-BSA together did not fully simulate the effect of native laminin, and were not essentially any better than the peptide p20-BSA alone in promoting neurite outgrowth, the effect of p31-BSA may be indirect and due to its growth factor-like properties [20].

Future work in this laboratory aims at characterizing the intermolecular interactions and co-operation between different domains of laminin that may be necessary to support neuronal development and survival. Synthetic peptides and their antibodies may be utilized in this approach, since our present results indicate, that a domain of laminin responsible for neurite outgrowth and neuronal attachment is largely independent of the tertiary structure or carbohydrate substitution of the native protein.

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