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Distinct heparin-binding and neurite-promoting properties of laminin isoforms isolated from chick heart

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SUMMARY

Laminin isolated from chick heart is composed of several heterotrimeric variants of 800 and 700 kDa. Here, we used monoclonal antibodies against chick laminin to purify different laminin isoforms from this mixture. Antibody 8D3 specifically removed laminin containing α2 chain from chick heart laminin preparations, leaving behind 700 kDa variants. Using antibody C4 against the laminin β2 chain, α2 chain containing variants were further separated into α2β1γ1 and α2β2γ1 laminin, respectively. Laminins containing α2 chain and recognized by antibody 8D3 are cross-shaped molecules. Their expression during embryogenesis is tightly regulated. In 5-day embryos staining with monoclonal antibody 8D3 is restricted to the dermamyotome. Older embryos (8 days) express α2 chain containing variants at myotendinous junction primordia of skeletal muscle, and only late in development these variants are generally expressed in skeletal and heart muscle basement membranes. The 700 kDa laminin variants contain β1, β2, and γ1 subunits affiliated with an immunologically distinct, shorter αx chain and appear to be T-shaped in the electron microscope. Whereas laminins with an α2 subunit bind to heparin, variants with the novel αx chain do not. Experiments using cultured sympathetic neurons showed that laminins with αx chain are less potent than α2 chain containing variants in promoting neurite outgrowth. In contrast, sympathetic neurons cannot discriminate between α2β1γ1 and α2β2γ1 laminin substrates, respectively, and show identical high rates of neurite formation.

Key words: laminin, nerve growth, heparin, extracellular matrix

INTRODUCTION

Laminins belong to a family of heterotrimeric glycoproteins abundant in basement membranes (Timpl and Brown, 1994). To date, at least eight different laminin chains have been characterized which can be classified into three groups according to their sequence homology and domain organization (Engel et al., 1994). Each laminin molecule is composed of one α, one β, and one γ chain which combine to give the 7 laminin isoforms described so far (Timpl and Brown, 1994; for nomenclature see Burgeson et al., 1994). The prototype, laminin 1, was isolated from the murine Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979). Its α1 chain (400 kDa), β1 and γ1 chains (220 kDa each) assemble to a cruciform structure with three short arms and a mostly triple helical long arm. A globular extension at the end of the long arm is contributed by the α1 chain (for review, see Beck et al., 1990). The α2 chain (merosin) was first found in human placenta (Ehrig et al., 1990). Hunter et al. (1989a) described the β2 chain (s-laminin) which is slightly smaller (190 kDa) than the β1 chain. These subunits are found in laminin 2 (α2β1γ1), laminin 3 (α1β2γ1) and laminin 4 (α2β2γ1) (Engvall et al., 1990). Anchoring filaments contain two other isoforms, laminin 5 (kalinin) and laminin 6 (K-laminin). Laminin 5 has distinct, smaller subunits (140-200 kDa) and is of the chain composition α3β3γ2 (Rousselle et al., 1991). Laminin 6 is composed of β1 and γ1 chains affiliated with an α chain related to α3 (Marinkovich et al., 1992). In laminin 7 the β1 chain is replaced by β2 (Timpl and Brown, 1994). Evidence exists for additional α and β chains (O'Rear, 1992; Tokida et al., 1990).

The diversity of the laminin family is also manifested in structure. As shown by electron microscopy, laminins 1-4 share a cruciform shape (Beck et al., 1990; Brown et al., 1994). Laminin 5 consists only of a long arm with terminal globules (Rousselle et al., 1991), whereas laminin 6 and 7 appear as T- or Y-shaped molecules missing one short arm (Marinkovich et al., 1992).

In vitro studies have shown that laminins can modulate cell shape, proliferation, differentiation, cell migration and nerve growth (Kleinmann et al., 1985). Laminin isoforms are differentially expressed and developmentally regulated (e.g. Ekblom et al., 1990; Engvall et al., 1990). This raises the question of whether they serve different functions during development. In particular laminins have been proposed to help guide neurites to their targets since they are strong promoters of neurite outgrowth in vitro (Baron-Van-Evercooren et al., 1982; Edgar et al., 1988; Tomaselli et al., 1993) and are present along certain neural pathways in development (Rogers et al., 1986). Hunter et al. (1989b, 1991) have generated recombinant fragments and synthetic peptides of the rat laminin β2 chain

that are selectively adhesive for motor neurons while inhibiting their neurite outgrowth. In addition, mice lacking the laminin β2 chain have abnormal neuromuscular junctions (Noakes et al., 1995). These experiments suggested that laminin variants may act on neurites in distinct ways and that they may be involved in nerve guidance and synapse formation (Hunter, 1989a,b, 1991).

In most reports published so far, laminin isoforms isolated from different species have been compared in functional assays (e.g. Engvall et al., 1992; Tomaselli et al., 1993). To address the question whether laminin isoforms indeed have different activities, we decided to separate and purify laminin isoforms from a single tissue source, i.e. chick heart (Brubacher et al., 1991), and to examine their influence on neurite growth by chick embryonic neurons. Laminin isoforms from chick heart can be divided into two groups, one of them cross-shaped, containing the α2 subunit and binding to heparin, and the other group T-shaped, with a novel αx chain not able to bind to heparin. We show the distribution of the α2 subunit containing variants during embryogenesis. In addition, we show that cultured neurons indeed discriminate between certain laminin isoforms but not between others.

MATERIALS AND METHODS

SDS-PAGE and immunoblotting

SDS-PAGE on 3-15% polyacrylamide gradient gels and immunoblotting were done as published (Koch et al., 1992).

Preparation of monoclonal antibodies

A monoclonal antibody (mAb) library against chick heart laminin was prepared as described (Brubacher et al., 1991). Balb/c mice were injected with chick heart laminin purified by mAb 9F10 affinity chromatography (Brubacher et al., 1991). 40 μg antigen in 200 μl TBS (Tris-buffered saline; 150 mM NaCl, 20 mM Tris/HCl, pH 7.4) was used for immunization and for boosting. Hybridoma supernatants were screened by immunoblotting against chick laminin and by immunofluorescence on cryosections. Selected hybridomas named 8D3 and 11B7 were cloned by serial dilution and rescreened by the same criteria. Resulting mAbs 8D3 and 11B7 are both IgG's. Immunoglobulins were purified from cell culture supernatant by precipitation with 40% saturated ammonium sulfate and adsorption to a goat anti-mouse IgG affinity column (a gift from Dr Manfred Brockhaus, Hoffmann-La Roche, Basel). The column was washed with TBS and eluted with 0.1 M glycine, pH 2.8. Antibody containing fractions were pooled and dialyzed against TBS.

Monoclonal antibody C4 developed by J. R. Sanes was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University, Baltimore, MD 21205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under contract N01-HD-2-3144 from the NICHD.

Polyclonal antibodies

An antiserum specific for the chick laminin α2 chain was generated as follows. 1 mg chick heart laminin purified by mAb 8D3 affinity chromatography (see below) was loaded on a preparative SDS-PAGE run under reducing conditions and blotted onto nitrocellulose (BA-85, Schleicher & Schuell). The band of 350 kDa was cut out, suspended in 0.2 ml Dulbecco's PBS (phosphate buffered saline) and sonicated on ice for homogenization. The sample was mixed with 0.2 ml Freund's complete adjuvant (Gibco) and injected intracutaneously into a rabbit. The rabbit was boosted one month later with the same preparation suspended in incomplete Freund's adjuvant. Antiserum 650 was obtained 14 days later.

Other antisera used in this study are antiserum 245 against whole chick heart laminin (Brubacher et al., 1991), and antiserum 321 directed against mouse laminin α2 chain (Paulsson and Saladin, 1989), obtained from Dr M. Paulsson (University of Koeln).

Purification of laminin from chick heart by lectin affinity chromatography

Laminin was extracted with EDTA from adult chick hearts as described (Brubacher et al., 1991). Extracts were concentrated 10-fold by ammonium sulfate precipitation (40% saturation), dialyzed against TBS and applied to a wheat germ agglutinin (WGA) Sepharose column (25 mg of WGA coupled to 5 g CNBr-activated Sepharose 4B, Pharmacia) equilibrated in TBS at 4°C. After washing with 400 ml buffer A (500 mM NaCl, 0.05% Triton X-100, 20 mM Tris/HCl, pH 7.4) the column was re-equilibrated in TBS and eluted with 10% *N*-acetyl-*D*-glucosamine in TBS. Laminin containing fractions were pooled and dialyzed against TBS.

Separation of laminin isoforms by sequential immunoaffinity chromatography

Samples of 10 mg mAb 11B7, 10 mg mAb 8D3, or 6 mg mAb C4, respectively, were coupled to CNBr-activated Sepharose 4B. WGA-purified chick heart laminin was applied to the mAb 11B7 column. After washing with buffer A the column was equilibrated in 150 mM NaCl and eluted with 150 mM NaCl, 0.1 M triethylamine, pH 11. All laminin isoforms bound to the mAb 11B7 column while unrelated contaminants were removed. Laminin containing fractions were neutralized by adding 1/10 volume of 1 M Tris-HCl, pH 6.8, pooled and dialyzed against TBS. Protein was then loaded on the mAb 8D3 affinity column which was washed and eluted as described above. The flowthrough was repassed twice over the same column to deplete it of mAb 8D3 immunoreactivity. Laminin retained on the mAb 8D3 column was further fractionated on a mAb C4 column using the same procedure as above. The concentration of laminin in the samples was determined by UV absorption at 280 nm using the extinction coefficient determined for mouse laminin 1, ε=0.746 ml mg^{-1} cm^{-1} (K. Beck, unpublished).

Heparin affinity chromatography

Laminins were dialyzed against TBS and loaded on a heparin-Sepharose column equilibrated in TBS. After washing with the same buffer, bound material was eluted in steps of 250 mM, 400 mM and 1 M NaCl, each in 20 mM Tris-HCl, pH 7.4.

Immunocytochemistry

Cryosections of 5-18-day-old chick embryos were prepared and stained as described (Brubacher et al., 1991). Antibodies 9F10 and 8D3 were diluted to 20 μg ml^{-1} in 0.5% bovine serum albumin in TBS. TRITC-labeled secondary goat anti-mouse IgG (Cappel) was diluted 1:200 in the same buffer.

Electron microscopy

Laminin samples were prepared for rotary shadowing and electron microscopy as published (Koch et al., 1992). For antibody localization by electron microscopy (Chiquet-Ehrismann et al., 1988), 80 μg ml^{-1} laminin purified by mAb 8D3 immunoaffinity chromatography were incubated with 25 μg ml^{-1} mAb 8D3 for 1 hour before processing.

Neuronal cell cultures

Sympathetic neurons were isolated from 10-day-old chick embryos and cultured in serum-free medium as described (Wehrle and Chiquet, 1990). To prepare culture substrata, round coverslips were coated with poly-L-lysine (Sigma, M_r=15,000), and then with 50 μl laminin at a concentration of 20-30 μg ml^{-1} (if not mentioned otherwise) as published (Wehrle and Chiquet, 1990). The amount of laminin bound

to the coverslip was determined by detaching the protein with SDS-sample buffer, and by comparing it to the applied amount on Coomassie stained polyacrylamide gels by densitometric scanning. Similar to published data (Buettner and Pittman, 1991), about 60% of the applied laminin adsorbed to the coverslip, independently of the isoform and of the coating concentration within the used range (data not shown).

Quantification of neurite outgrowth

To measure the rate of neurite outgrowth by sympathetic neurons, two randomly chosen fields (3.5 mm^2 each) per culture well were photographed on an inverted microscope at different times of incubation. From projected negatives cell numbers and neurite lengths per field were measured using a graphics tablet. In these experiments, all processes distinguishable as neurites were counted. Data are expressed as the sum of neurite lengths divided by the number of sprouting cells per counted field. Only fields containing 70 or more sprouting neurons were evaluated. At least six measured values were averaged per time point and substrate and standard deviations were calculated.

In addition, the distribution of neurite lengths per single neuron at 1.5 hours in culture was analyzed similarly to Chang et al. (1987). Here, neurites were counted only if longer than one cell diameter and if they had not contacted other neurites. Data are plotted as the percentage of neurons (vertical axis) with neurites longer than a given length (horizontal axis). The non-parametric Wilcoxon test was used to determine whether neurite lengths were statistically different between substrates. $P<0.05$ was considered to be significant.

RESULTS

Strategy to separate chick laminin isoforms by sequential mAb affinity chromatography

For clarity, the procedure used to separate chick laminin isoforms is shown schematically in Fig. 1 and the evidence is described in the following paragraphs.

Partially purified chick heart laminin was passed over three affinity columns coupled with different chain specific antibodies. First, mAb 11B7 against chick laminin γ1 chain adsorbed all isoforms present in chick heart. A mAb 8D3 column then specifically retained isoforms with α2 chain (called $\alpha2^+$ laminin), whereas isoforms without this chain were found in the flowthrough ($\alpha2^-$ laminin). In the third step, $\alpha2^+$ laminin was passed over a column coupled with mAb C4 against the laminin β2 chain, yielding bound laminin 4 (α2β2γ1) and unbound laminin 2 (α2β1γ1).

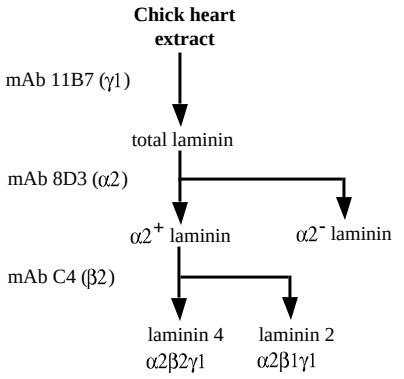

Fig. 1. Scheme showing the purification of chick laminin isoforms by sequential affinity chromatography. The laminin chains recognized by the antibodies and the chain composition of the isoforms are indicated.

Purification of total chick heart laminin by mAb 11B7

EDTA extracts from chick heart (Fig. 2a) were passed over WGA-Sepharose. Eluted fractions were enriched considerably in three bands of high molecular mass on non-reducing SDS-PAGE (Fig. 2b). These bands were confirmed to be laminins by immunoblotting using antiserum 245 against chick heart laminin (not shown; cf. Brubacher et al., 1991). This material was loaded on a Sepharose column coupled with mAb 11B7. This antibody was used instead of the previously described mAb 9F10 (Brubacher et al., 1991) since both antibodies recognize all laminin variants in chick heart but mAb 11B7 proved to be superior for affinity purification. Antibody 9F10 is directed against the major 205 kDa band of chick heart laminin (Brubacher et al., 1991). This polypeptide is also recognized by an antiserum against recombinant human γ1 chain (obtained from Dr P. W. Taylor, Ciba, Horsham, UK; not shown). In addition, laminin purified by either mAb 9F10 or 11B7 affinity chromatography contains 350 kDa α2 chain and 190 kDa β2 chain (see below and Brubacher et al., 1991). Therefore, mAbs 9F10 and 11B7 must be directed against the chick laminin γ1 chain. Laminins adsorbed quantitatively to mAb 11B7-Sepharose and eluted fractions appeared electrophoretically pure (Fig. 2c), apart from a minor band migrating at 66 kDa. In some preparations a band of 150 kDa could be detected and was identified as nidogen by immunoblotting (not shown).

Purification of laminin with 350 kDa α2 chain by mAb 8D3

Of the three chick heart laminin bands visible on SDS-PAGE under non-reducing conditions (Fig. 2c), the 800 and 700 kDa bands are due to distinct populations of single molecules, whereas the >1,000 kDa band is likely to represent covalently linked dimers (Brubacher et al., 1991). To separate variants from this mixture, we generated a new mAb library and screened for antibodies able to discriminate between the three non-reduced heart laminin bands. One mAb, termed 8D3, rec-

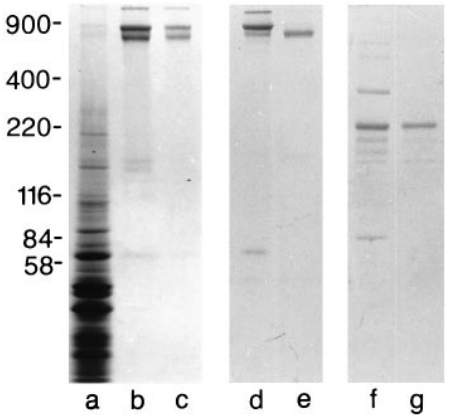

Fig. 2. Sequential purification of chick heart laminin variants by affinity chromatography. Coomassie stained SDS-PAGE; lanes a-e without, lanes f,g with reduction. (a) TBS/EDTA extract of chick heart; (b) laminin purified by WGA-affinity chromatography; (c) laminin eluted from mAb 11B7-Sepharose; (d,f) material bound to mAb 8D3-Sepharose; (e,g) laminin in flowthrough after affinity chromatography to mAb 8D3. Molecular mass standards in kDa are indicated. 900, 400 and 220 denote molecular masses of intact laminin 1, α1 and β1/γ1 chains of laminin 1, respectively.

ognized predominantly the 800 kDa band and only faintly labeled the two other bands on immunoblots (see below, Fig. 4A,c). Antibody 8D3 was coupled to Sepharose and purified chick heart laminin was applied to the column. The eluate was strongly enriched in 800 kDa band as seen on non-reducing SDS-PAGE (Fig. 2d). In addition, the band migrating at 66 kDa was visible.

The reduced polypeptide pattern of laminin eluted from the mAb 8D3 column (Fig. 2f) was more complex than that of mouse laminin 1 (400 kDa and 220 kDa). The major 205 kDa

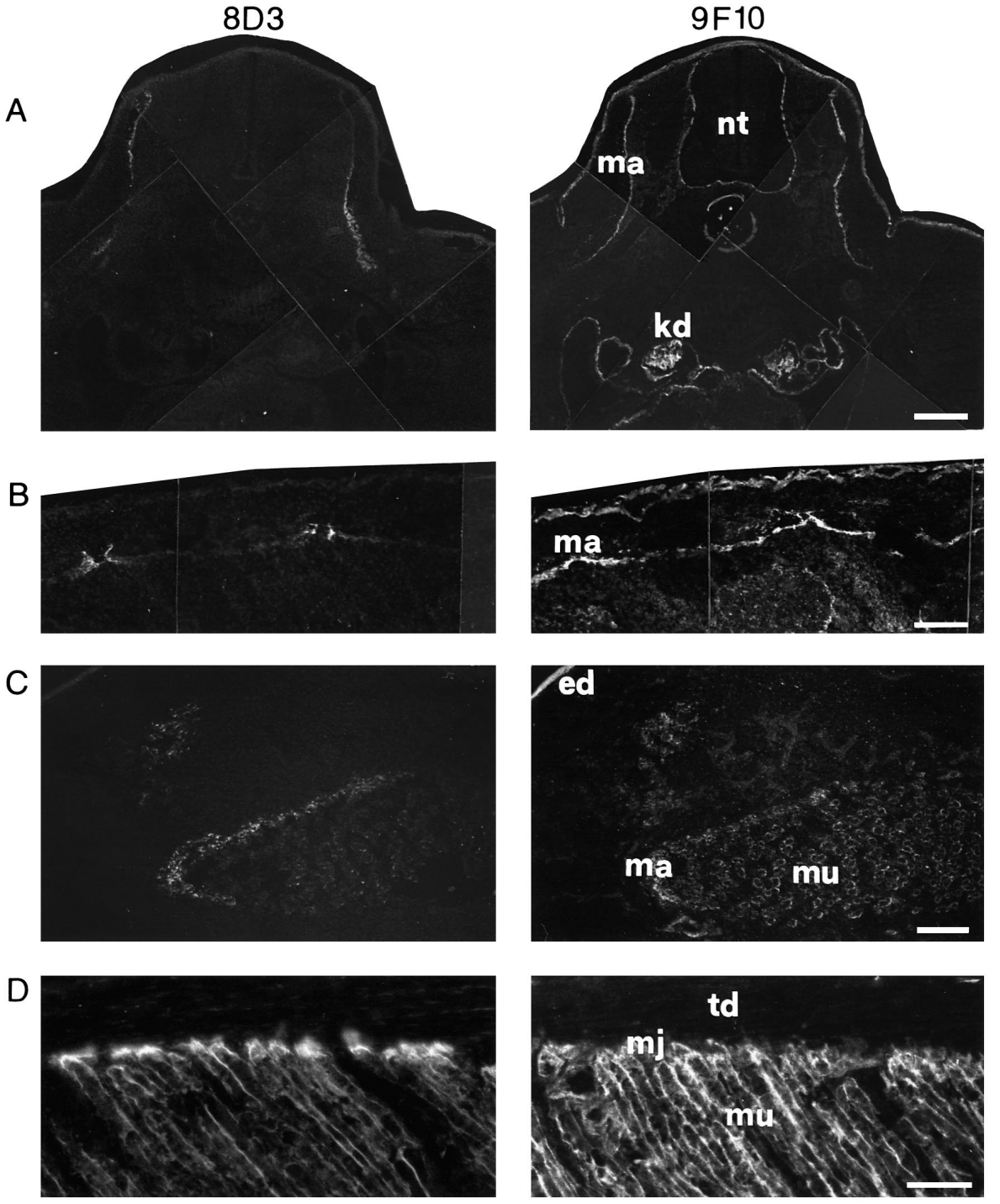

Fig. 3. Distribution of $\alpha 2^+$ laminin during chick embryonic development. Sections were labeled with mAb 8D3 recognizing $\alpha 2^+$ laminin (left panel) or with mAb 9F10 against the γ1 chain (right panel). (A) Cross-section through a 5-day embryo at the level of the wing buds; (B) sagittal section through the trunk region of a 5-day embryo (dorsal is up, anterior is to the right); (C) longitudinal section of an 8-day wing (dorsal is up, proximal is to the left); (D) section through an 18-day leg muscle. Note that in young embryos (A-C) mAb 8D3 labels only muscle fiber attachment sites. ed, epidermis; dg, dorsal root ganglion; kd, kidney; ma, muscle fiber attachment site; mj, myotendinous junction; mu, muscle; nt, neural tube; td, tendon. Bars: 50 μm (A), 25 μm (B), 100 μm (C), 50 μm (D).

band contains the γ1 chain and probably other subunits, whereas the 350 kDa band has been identified immunologically as the α2 chain homologue in the chick (Brubacher et al., 1991). Apart from these, minor polypeptides of higher and lower molecular mass were present. The 600 and 500 kDa bands are due to non-reducible crosslinks between two laminin subunits (Brubacher et al., 1991; Paulsson and Saladin, 1989). The 150 kDa polypeptide was identified as nidogen by immunoblotting (not shown). The band at 66 kDa (non-reduced) or 80 kDa (reduced) probably corresponds to a laminin α2 chain fragment (Ehrig et al., 1990; Brown et al., 1994). The identity of a 190 kDa band will be addressed below.

Apparently, whereas most of the laminin of 800 kDa adsorbed to the mAb 8D3 column, only a 700 kDa band (non-reduced) was present in the flowthrough (Fig. 2e). The polypeptide pattern obtained upon reduction of this species was much simpler. Only three bands of 205 kDa, 190 kDa and 150 kDa (nidogen, not shown), but no polypeptide of a size similar to the laminin α1 or α2 chains were visible (Fig. 2g). Thus, by immunoaffinity chromatography to mAb 8D3 we were able to separate two laminin subpopulations which are distinguished by the presence or absence of a 350 kDa α2 subunit. We therefore call isoforms retained by the mAb 8D3 column $\alpha2^+$ laminin, and isoforms not retained $\alpha2^-$ laminin.

Restricted distribution of $\alpha2^+$ laminin in the chick embryo

The distribution of $\alpha2^+$ laminin during chick embryonic development was monitored by immunofluorescence using mAb 8D3 and found to be very restricted. Fig. 3A,B shows that in 5-day embryos only the myotendinous junction primordia of the dermamyotome are stained by mAb 8D3. In contrast, mAb 9F10 which recognizes most laminin isoforms present in the chick (Brubacher et al., 1991) labeled all basement membranes at this stage (Fig. 3A,B). Embryos at 8-9 days show staining at myotendinous junctions of skeletal muscles of the limbs (Fig. 3C). The remainder of muscle fiber basement membranes apparently lack $\alpha2^+$ laminin but are stained with mAb 9F10 manifesting the presence of other laminin variants. Between this and later stages a change in the distribution pattern of $\alpha2^+$ laminin occurs. At embryonic day 18, the entire skeletal muscle basement membrane is also stained by mAb 8D3 (Fig. 3D). Nevertheless, $\alpha2^+$ laminin remains enriched at myotendinous junctions. In cardiac muscle basement membranes, $\alpha2^+$ laminin could be detected shortly before birth (not shown). Although a few other structures (e.g. the epithelial lining of the gizzard) were found to be labeled, most tissues were negative (not shown).

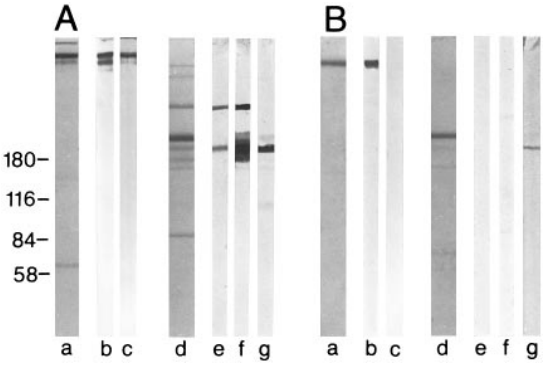

Fig. 4. Immunological characterization of chick heart laminin variants. (A) laminin bound to mAb 8D3 ($\alpha2^+$ laminin); (B) flowthrough of mAb 8D3 column ($\alpha2^-$ laminin). SDS-PAGE under non-reducing (a-c) or reducing (d-g) conditions. Lanes a,d are stained with Coomassie, the other lanes immunoblotted with the following antibodies: lane b, mAb 9F10 recognizing chick laminin γ1 chain; lane c, mAb 8D3; lane e, antiserum 321 against mouse laminin α2 chain; lane f, antiserum 650 raised against chick laminin α2 subunit; lane g, mAb C4 against rat laminin β2 chain. Positions of molecular mass standards are indicated in kDa.

Immunological characterization of $\alpha2^+$ and $\alpha2^-$ laminin

To obtain information about the subunit composition of $\alpha2^+$ and $\alpha2^-$ laminin populations, we tested their reactivity with different antibodies on immunoblots (Fig. 4). Both laminin populations were strongly labeled by mAb 9F10 against the chick γ1 chain (Fig. 4A,b, and B,b). As expected, mAb 8D3 only recognized $\alpha2^+$ laminin (Fig. 4A,c) but not $\alpha2^-$ laminin (4B,c). We also tested other antibodies that are specific for distinct laminin subunits on reducing gels. Antiserum 321 against the mouse α2 laminin subunit (Paulsson and Saladin, 1989) labeled the 350 kDa as well as a 190 kDa polypeptide present in $\alpha2^+$ laminin (Fig. 4A,e). The same pattern was observed with antiserum 650 which we obtained by immunization with the 350 kDa α2 chain of chick heart laminin (Fig. 4A,f). The strong reactivity of antisera 650 and 321 with a 190 kDa polypeptide indicates a major proteolytic fragment of the α2 subunit. Nothing was recognized by these antisera in $\alpha2^-$ laminin (Fig. 4B,e,f). The 190 kDa region in $\alpha2^+$ laminin was also strongly labeled by monoclonal antibody C4 which is directed against the rat and cross-reacts with the chick β2 chain (Fig. 4A,g). The 190 kDa polypeptide present in $\alpha2^-$ laminin also reacted weakly with mAb C4 (Fig. 4B,g).

In conclusion, both $\alpha2^+$ and $\alpha2^-$ laminin possess a common 205 kDa γ1 subunit. Both populations also have β2 chain, but might contain other β chains (such as β1) as well. $\alpha2^-$ laminin must possess an immunologically distinct αx chain which is smaller than the α1 or α2 chains.

Structural differences between $\alpha2^+$ and $\alpha2^-$ laminin

The two laminin populations separated by mAb 8D3 were visualized by rotary shadowing. The $\alpha2^+$ laminin molecules appear as cross-shaped particles similar in size and shape to laminins 1-4 (Fig. 5A,B). In contrast, $\alpha2^-$ laminin consists of T-shaped molecules lacking one of the short arms, similar to the structure of laminin 6 from human keratinocytes (Marinkovich et al., 1992) (Fig. 5D,E). In addition, the terminal globule consisting of the G domains (Beck et al., 1990) seems to be slightly smaller than the one of $\alpha2^+$ laminin.

To reveal the epitope of mAb 8D3, $\alpha2^+$ laminin was incubated with this antibody, rotary shadowed and viewed in the electron microscope. Antibodies were found to bind to the lower end of the long arm (Fig. 5C). Since such a triple coiled coil structure is present in both $\alpha2^+$ and $\alpha2^-$ laminin, this is further evidence that the two laminin populations contain distinct α chains, rather than that the T-shaped molecules are proteolytic products of cross-shaped $\alpha2^+$ laminin.

Differential heparin binding properties of $\alpha2^+$ and $\alpha2^-$ laminin

Additional evidence for the heterogeneity of chick heart

laminins came from heparin binding studies (Fig. 6). After passing mAb 11B7 purified laminin over heparin-Sepharose under physiological salt conditions, the flowthrough contained mainly the 700 kDa variant whereas the 800 kDa species was eluted with 250 mM NaCl (Fig. 6, left panel). When isolated $\alpha2^+$ laminin was applied to the heparin column, the flowthrough was devoid of protein and all material was eluted with 250 mM NaCl (Fig. 6, center panel). In the case of $\alpha2^-$ laminin, no protein at all bound to the heparin-Sepharose in 150 mM NaCl and all laminin was found in the flowthrough (Fig. 6, right panel). This result points to a functional difference between the two laminin populations, related to the presence or absence of $\alpha2$ chain.

Separation of $\alpha2^+$ laminin into distinct isoforms by mAb C4 affinity chromatography

The $\alpha2^+$ and $\alpha2^-$ laminin populations are still mixtures of more than one isoform. To further explore the composition of $\alpha2^+$

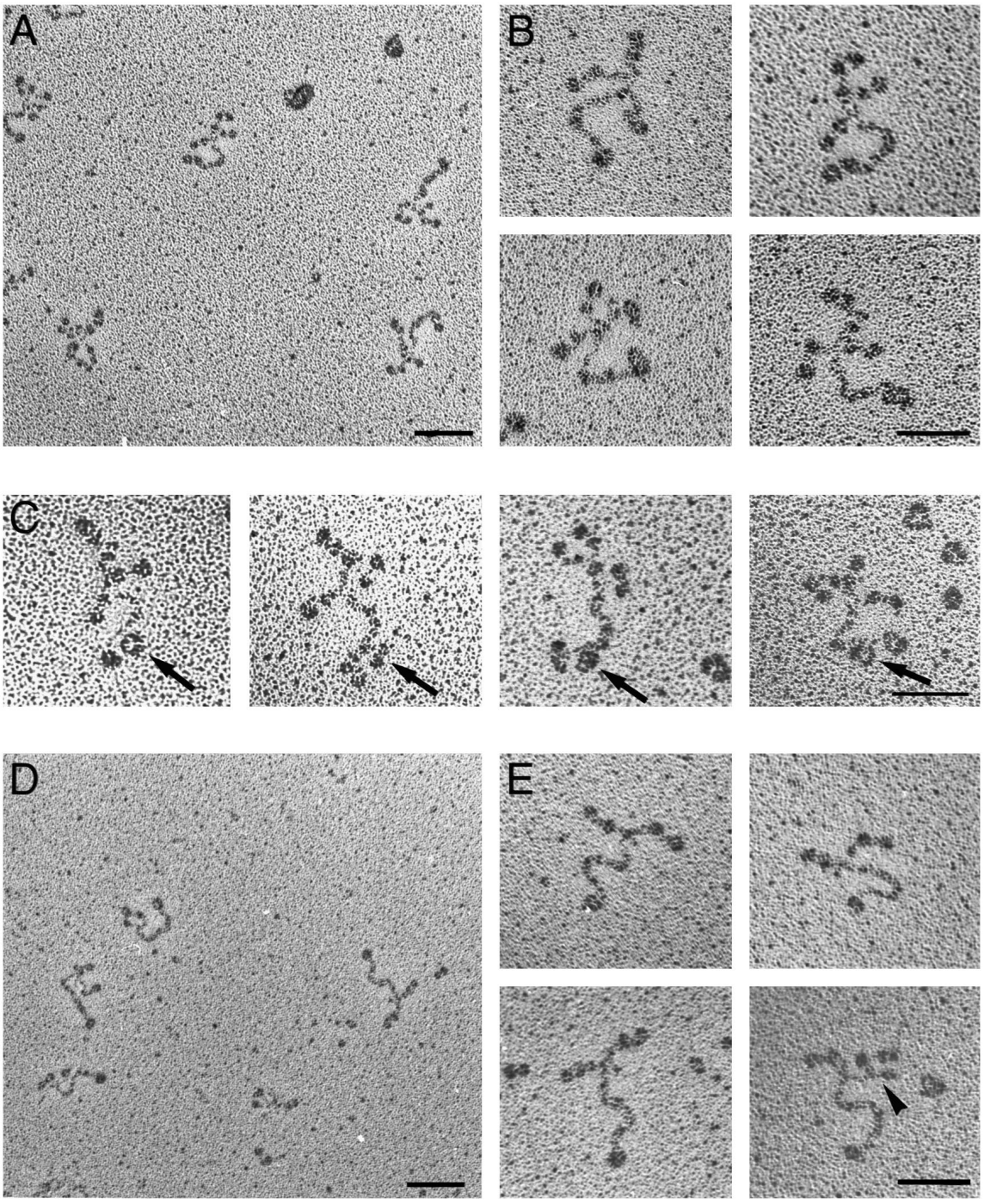

Fig. 5. Electron micrographs of rotary shadowed chick laminin variants $\alpha2^+$ and $\alpha2^-$, and epitope mapping of mAb 8D3. (A,B) $\alpha2^+$ laminin; (C) $\alpha2^+$ laminin labeled with mAb 8D3, arrows point to bound antibody; (D,E) $\alpha2^-$ laminin, arrowhead indicates a bound nidogen. Bars: A,D, 100 nm; B,C,E, 50 nm.

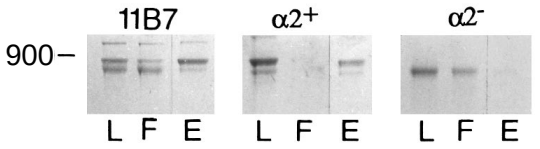

Fig. 6. Differential heparin binding of $\alpha2^+$ and $\alpha2^-$ laminin; SDS-PAGE under non-reducing conditions. Laminin purified by mAb 11B7 (11B7), $\alpha2^+$ laminin ($\alpha2^+$), or $\alpha2^-$ laminin ($\alpha2^-$), respectively, were applied to a heparin-Sepharose column equilibrated in TBS. The column was washed with TBS and eluted with 0.25 M NaCl. L, sample loaded on the heparin-Sepharose; F, flowthrough; E, eluted protein. Molecular mass standard is indicated (kDa).

laminin, we used mAb C4 against the β2 chain as an affinity reagent (Fig. 7). When purified $\alpha2^+$ laminin was applied to a Sepharose column coupled with mAb C4, most of the protein was found in the flowthrough and only a small amount could be eluted from the column (Fig. 7, Coom). Eluted laminin consisted of two non-reduced bands which were both of slightly smaller molecular mass than the 800 kDa laminin band present in the flowthrough. As expected, both the flowthrough and the eluate were recognized by mAb 8D3 on immunoblots (Fig. 7, 8D3). Upon reduction, the 205 kDa band was the major species in the flowthrough whereas the eluate contained as much 190 kDa as 205 kDa polypeptide (Fig. 7, silver). By immunobloting, both variants separated by mAb C4 reacted with anti-α2 chain antibody 650 (Fig. 7, 650). Expectedly, only laminin bound to the mAb C4 column contained β2 chain while this subunit was absent in the flowthrough (Fig. 7, C4). Both variants possessed γ1 chain (Fig. 7, 9F10). These findings indicate that the protein bound to the mAb C4 column is laminin 4 of the chain composition α2β2γ1. Because it has been purified by mAbs against three different subunits, it should be a homogenous isoform. Its size heterogeneity on non-reduced gels (Fig. 7, Coom) is likely to be due to partial cleavage of the 350 kDa α2 chain into a 190 kDa fragment (Fig. 7, 650). The flowthrough of the mAb C4 column contains an isoform with a different β subunit, most likely this is α2β1γ1 laminin (laminin 2; see Discussion).

Neurite outgrowth promotion by the purified laminin variants

In a first step to investigate whether different laminin isoforms might have distinct functions in neurogenesis, we compared the isolated preparations for their ability to promote neurite outgrowth by sympathetic neurons from 10-day-old chick embryos. Coverslips were coated with either $\alpha2^+$ laminin, $\alpha2^-$ laminin, laminin 2, or laminin 4, respectively. Neurons were plated on these substrates and their average rate of neurite formation was measured. All chick laminin variants promoted rapid neurite outgrowth, but $\alpha2^-$ laminin was consistently less potent than $\alpha2^+$ laminin (Fig. 8A,B). The largest difference was observed at three hours, and after twelve hours neurites grown on $\alpha2^+$ laminin were still significantly longer than on $\alpha2^-$ laminin ($P<0.05$). This difference was not due to different concentrations of laminin variants present on the coverslips (see Materials and Methods). Saturating coating conditions were used, and up to 4-fold dilution of the applied protein samples did not markedly affect the rate of neurite formation (not shown).

To assess by which extent laminin 2 or laminin 4 contribute to the neurite promoting activity of $\alpha2^+$ laminin, purified isoforms separated over the mAb C4 column were analyzed in similar, independent experiments. We could not detect any significant difference between these two isoforms (Fig. 8C). Likewise, 4-fold dilution of applied samples did not change growth rate or length of neurites (not shown).

In order to obtain more concise information on the distribution of neurite lengths per cell on different laminins, an analysis similar to that of Chang et al. (1987) was performed (Fig. 9). Here, neurite lengths of individual neurons were measured at 1.5 hours of incubation. A clear difference ($P<0.05$) was again observed for $\alpha2^+$ laminin versus $\alpha2^-$ laminin substrates (Fig. 9A), whereas comparison of laminin 2 with laminin 4 showed equal distributions of neurite lengths (Fig. 9B).

DISCUSSION

Laminins are strong promoters of neurite outgrowth in vitro and are thought to be involved in axon guidance in development (Anton et al., 1994; Baron-Van-Evercooren et al., 1982; Edgar et al., 1988). However, laminin expression is not confined to pathways along which neurons grow in the embryo (Brubacher et al., 1991). It has therefore been suggested that laminin is a permissive rather than a guiding substrate for growing axons (Bixby and Harris, 1991). Recent evidence for

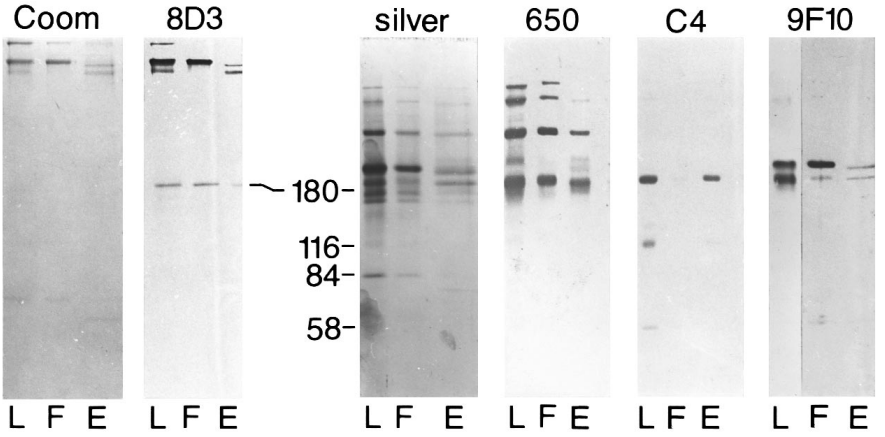

Fig. 7. Separation of $\alpha2^+$ laminin into laminin 2 and laminin 4 isoforms by mAb C4 affinity chromatography, and immunoblot of separated variants. L, $\alpha2^+$ laminin applied to the mAb C4 column; F, flowthrough; E, bound protein. Coom: Coomassie stained SDS-PAGE, non reduced; 8D3, immunoblot probed with mAb 8D3; silver, silver stained SDS-PAGE, reduced; 650, immunoblot with anti-laminin α2 antiserum 650; C4, immunoblot using anti-laminin β2 mAb C4; 9F10, immunoblot probed with anti-laminin γ1 mAb 9F10. In this experiment, antiserum 650 labeled the 350 and 190 kDa α2 bands as well as non-reducible crosslinks of 600 and 500 kDa as also observed for mouse heart laminin (Paulsson and Saladin, 1989). Molecular mass standards are indicated in kDa.

the existence of multiple laminin isoforms (Ehrig et al., 1990; Hunter et al., 1989a; Marinkovich et al., 1992; Rousselle et al., 1991) renewed interest in the possibility that at least some of them might have specific effects on the steering of growing axons. Indeed, Hunter et al. (1989a,b, 1991) found that the laminin β2 subunit is enriched in neuromuscular junctions (among other places), and they identified a sequence present in this chain which allowed attachment but inhibited neurite growth by motor neurons. They argued that the local presence of laminin β2 chain might be a 'stop signal' for growing axons. So far, these experiments have been performed with recombinant and synthetic peptides and not with intact trimeric laminin containing β2 chain. In parallel, commercially available laminin variants have recently been compared with respect to their neurite-promoting activity in vitro, and some differences were found (Calof et al., 1994; Tomaselli et al., 1993). However, these laminins were derived from different species, and commercial human placenta 'merosin' is a mixture of at least two laminin isoforms, 2 and 4 (Engvall et al., 1990). Since the chick embryo is very well suited to study neurogenesis (Lance-Jones and Landmesser, 1981), we decided to purify laminin isoforms from this species and to study their activity on chick neurons.

The heterotrimeric nature of laminins makes it difficult to purify single isoforms from a mixture. Brown et al. (1994) used anion exchange chromatography to separate distinct laminin species. A second possibility is offered by sequential immunoaffinity chromatography to subunit specific antibodies (Engvall et al., 1990). By employing chick-specific mAbs, we adopted this technique to fractionate a heterogeneous chick

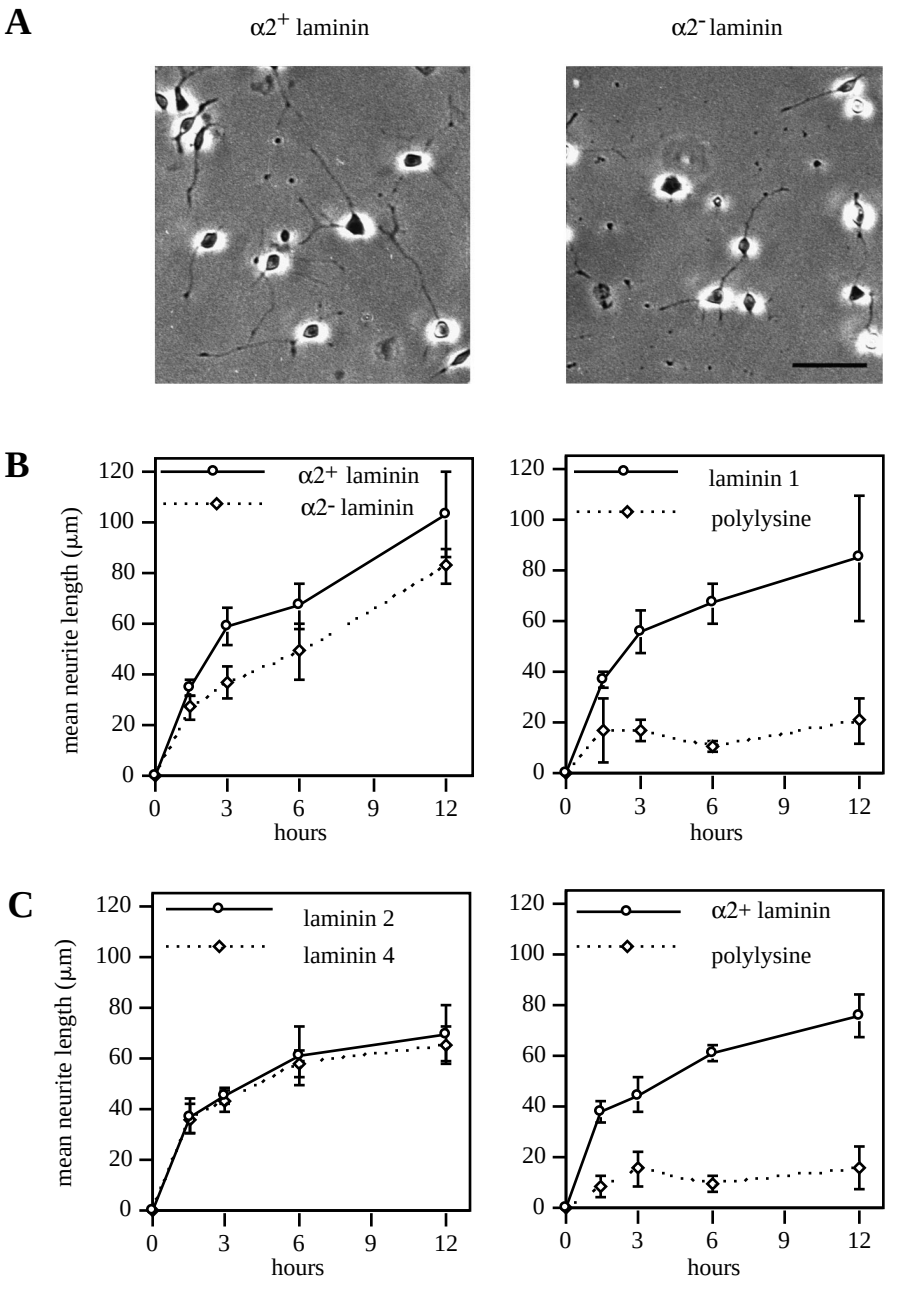

Fig. 8. Process formation by sympathetic neurons cultured on laminin isoforms. (A) Photos after 3 hours of incubation on chick $\alpha2^+$ laminin and $\alpha2^-$ laminin, respectively. Bar, 50 μm. (B,C) Kinetics of neurite outgrowth by sympathetic neurons plated on chick laminin variants. (B) Comparison of chick $\alpha2^+$ laminin with $\alpha2^-$ laminin (left graph); growth on mouse laminin 1 and on polylysine in the same experiment is shown for comparison (right graph). Standard deviations are indicated. For all timepoints a significant difference ($P<0.05$) in neurite length was evaluated. (C) Comparison of laminin 2 with laminin 4 is shown at left and growth on chick $\alpha2^+$ laminin and on polylysine in the same experiment at right.

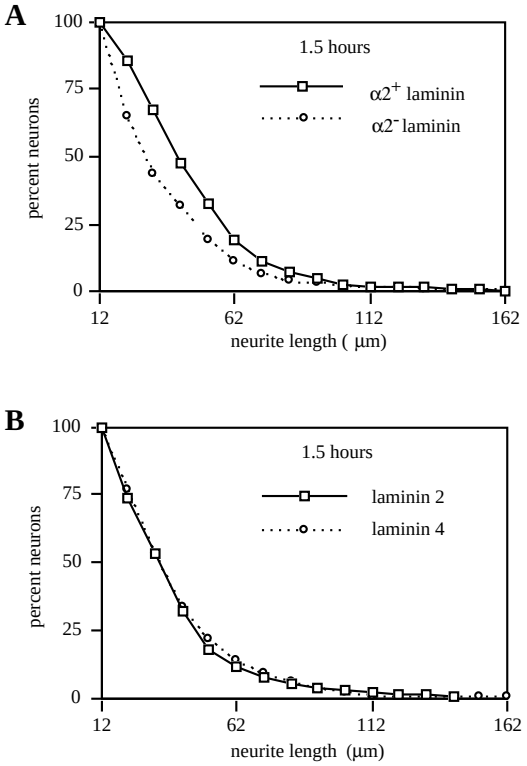

Fig. 9. Comparison of individual neurite lengths of sympathetic neurons cultured on chick laminin variants at 1.5 hours of incubation. Data are shown as percentage of neurons (*y*-axis) with neurites longer than a given length (*x*-axis). (A) Neurite lengths on $\alpha2^+$ laminin versus $\alpha2^-$ laminin; (B) laminin 2 compared with laminin 4; a significant difference ($P<0.05$) was obtained for the curves in A but not for those in B. Number of measured neurons on various substrates: $\alpha2^+$ laminin, 263; $\alpha2^-$ laminin, 276; laminin 2, 400; laminin 4, 364.

laminin mixture (Brubacher et al., 1991) into distinct isoforms. Laminin variants were then compared in terms of expression, structure and neurite-promoting activity.

Published data on the embryonic expression of laminin α2 subunit are somewhat contradictory. By immunohistochemistry, the protein was only detected postnatally in the mouse (Leivo and Engvall, 1988). In contrast, Vuolteenaho et al. (1994) found α2 chain mRNA to be present in various human fetal tissues. By staining with mAb 8D3, chick $\alpha2^+$ laminin shows an interesting distribution during embryonic development, being specifically located at myotendinous junction primordia in young embryos and only appearing at later stages in the basement membrane along skeletal muscle fibers where the α2 chain is known to be present in the adult (Sanes et al., 1990). Although mAb 8D3 also stained some other structures, most tissues were not labeled. Only shortly before birth $\alpha2^+$ laminin could be detected in cardiac muscle basement membranes.

When viewed by electron microscopy, chick laminin containing an α2 chain appeared to be mostly cross-shaped, while molecules lacking one short arm were occasionally observed. Laminin 4 (α2β2γ1) from adult heart in particular consisted of two species of 800 kDa and 700 kDa, in contrast to laminin 4 isolated from human placenta which is homogeneous in size (Brown et al., 1994). In chick $\alpha2^+$ laminin, antisera specific for the 350 kDa α2 subunit recognized a major fragment of 190 kDa in size which might explain the truncated molecules. A similar fragment of the α2 chain has been observed in murine and bovine heart laminin (Paulsson et al., 1991). The fraction of chick $\alpha2^+$ laminin which does not bind to the mAb C4 column is homogeneous in size (800 kDa). In analogy to laminin from bovine heart (Lindblom et al., 1994), it most likely contains β1 instead of β2 chain, and therefore would represent chick laminin 2 (α2β1γ1).

Laminins have multiple binding sites for other ECM molecules and for cell surface receptors. For example, a major heparin binding site is located on the C-terminal subdomains G4 and G5 of the α1 chain (Ott et al., 1982; Yurchenco et al., 1993). Brown et al. (1994) found that human laminins 2 and 4 only partially adsorb to heparin. In contrast, we found that chick $\alpha2^+$ laminin binds quantitatively to heparin in 150 mM NaCl.

In terms of promoting neurite growth by cultured chick sympathetic neurons, chick $\alpha2^+$ laminin is as active as mouse laminin 1. Interestingly, these as well as DRG neurons (R. Brandenberger, unpublished observation) also showed identical rates of neurite formation when cultured on either separated chick laminin 2 or 4. This is in apparent contrast to the postulated inhibitory activity of the β2 chain (present in laminins 3 and 4) for neurite outgrowth by motor neurons (Hunter et al., 1991). It has very recently been claimed that recombinant β2 chain peptides specifically inhibit growth by motor but not other neurons (Porter et al., 1995). We are currently testing the activity of native laminin 4 for motor neurons.

The smaller (700 kDa) $\alpha2^-$ laminin from chick heart appears T- or Y-shaped in the electron microscope. It still possesses a globular domain at the end of the long arm, and this structure is known to be contributed by α chains (Beck et al., 1990). The $\alpha2^-$ laminin must therefore contain another αx subunit of smaller size whose identity remains obscure, since on reduced gels no additional band was visible compared to $\alpha2^+$ laminin. T- or Y-shaped laminins have been described earlier (Edgar et al., 1988; Marinkovich et al., 1992). Laminin 5 (kalinin) and laminin 6 (K-laminin) have truncated α chains lacking short arm structures (Marinkovich et al., 1992; Rousselle et al., 1991). The relationship of $\alpha2^-$ laminin to laminin 5 was tested on immunoblots using an antiserum specific for human kalinin (a kind gift from Dr R. Burgeson, Boston, MA; data not shown). This antibody failed to recognize $\alpha2^-$ laminin but this could be due to species differences. However, since laminins 5 and 6 have so far only been found in skin and fetal membranes (Marinkovich et al., 1992; Rousselle et al., 1991), the αx chain from chick heart might be novel.

In contrast to $\alpha2^+$ laminin, $\alpha2^-$ laminin is not able to bind heparin. This finding reflects functional diversity of the unknown αx chain compared to the α2 subunit. However, it cannot be ruled out completely that $\alpha2^-$ laminin has lost its heparin binding properties due to limited proteolysis which, however, must be specific for its αx chain. Functional diversity was also manifested in neurite outgrowth experiments where $\alpha2^-$ laminin was significantly less active than $\alpha2^+$ laminin. This shows that sympathetic neurons can discriminate between these different laminin variants. It will be interesting to characterize the αx chain in chick heart laminin, and to see whether the lack of a strong heparin binding site in isoforms containing this chain is responsible for their decreased activity in neurite outgrowth.

In summary, our data show that various isoforms isolated from a single chick tissue show slight functional differences when tested on neurons from the same species. Whether such differences would be sufficient to steer growing axons in vivo remains to be investigated.

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