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Laminin-121—Recombinant expression and interactions with integrins

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ABSTRACT

Laminin-121, previously referred as to laminin-3, was expressed recombinantly in human embryonic kidney (HEK) 293 cells by triple transfection of full-length cDNAs encoding mouse laminin α1, β2 and γ1 chains. The recombinant laminin-121 was purified using Heparin-Sepharose followed by molecular sieve chromatography and shown to be correctly folded by electron microscopy and circular dichroism (CD). The CD spectra of recombinant laminin-121 were very similar to those of laminin-111 isolated from Engelbreth-Holm-Swarm tumor (EHS-laminin) but its T_m value was smaller than EHS-laminin and recombinant lamnin-111 suggesting that the replacement of the β chain reduced the stability of the coiled-coil structure of laminin-121. Its binding to integrins was compared with EHS-laminin, laminin-3A32 purified from murine epidermal cell line and recombinantly expressed laminins-111, -211 and -221. Laminin-121 showed the highest affinity to α6β1 and α7β1 integrins and furthermore, laminin-121 most effectively supported neurite outgrowth. Together, this suggests that the β2 laminins have higher affinity for integrins than the β1 laminins.

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1. Introduction

Laminins represent a protein family which are mainly localized in basement membranes and are involved in cell-matrix and various homo- and hetero-typic interactions. They consist of three genetically distinct chains, referred to as α-, β- and γ-chains with five α-, three β- and three γ-chains so far identified. Sixteen isoforms are described to date and according to the current nomenclature, laminins are termed based on from which α-, β- and γ-chains they are assembled (Aumailley et al., 2005). Laminins are multidomain proteins composed of different domains/modules which are also found in other extracellular matrix proteins and distinct domains in laminins are shown to be involved in important interactions for development and tissue homeostasis (Hohenester and Engel, 2002; Sasaki et al., 2004; Aumailley et al., 2005).

Laminin-121 is one of the α1 chain containing laminins and the other isoform is laminin-111, which is the first identified laminin isolated from the EHS tumor (Timpl et al., 1979) and from teratocarcinoma cells (Chung et al., 1979). The α1 chain is detected at the 2-cell stage of the pre-implantation mouse embryo and is subsequently expressed mainly in many epithelial tissues during organogenesis (Dziadek and Timpl, 1985). Its expression becomes more restricted in adult tissues (Ekblom et al., 2003). Laminin-111 is a major laminin found at early stages of embryogenesis and mouse embryos lacking laminin β1 and γ1 chain are devoid of basement membranes and do not survive beyond E 5.5 (Smyth et al., 1999; Miner et al., 2004). However, laminin α1-deficient embryos can form basement membranes, and develop further due to partial compensation by the α5 chain but die by E 7 (Miner et al., 2004). By contrast, mice lacking the laminin β2 chain die postnatally between days P 15 and P 30 and have serious defects in neuromuscular synapses and glomerular filtration (Noakes et al., 1995a,b). Biochemical and biophysical analyses on laminin have concentrated on laminin-111 from EHS tumor due to its availability in large quantity. Compared to laminin-111, almost nothing is known about laminin-121, previously referred as to laminin-3. The

Abbreviations: HEK, human embryonic kidney; CD, circular dichroism; EHS-laminin, laminin-111 isolated from Engelbreth-Holm-Swarm tumor; LN, laminin N-terminal; LG, laminin globular.

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existence of laminin-121 was demonstrated biochemically in human placenta (Champliaud et al., 2000). However, the studies by Durbeej et al. (1996) have suggested that the expression of the α1 and the β2 chains is mutually exclusive in rodent. It is therefore uncertain whether laminin-121 exists in rodent tissues. One of the reasons why distribution of laminin-121 may have been underestimated is that expression studies before the discovery of other laminin chains/isoforms have been misinterpreted. The monoclonal antibody 4C7, previously thought to recognize the α1 chain, has been used extensively for immunohistochemical studies of the α1 chain in human tissues including placenta (Engvall et al., 1990; Korhonen and Virtanen, 1997). In fact, the antibody 4C7 recognizes the α5 chain (Tiger et al., 1997), thus demonstrating the existence of laminin-521 instead of laminin-121 in many tissues. Furthermore, although restricted expression of the β2 chain in mouse kidney and skeletal muscle has been reported using monoclonal antibodies (Noakes et al., 1995a,b; Patton et al., 1997), polyclonal antibodies have demonstrated widespread expression of the β2 chain and also the distinct presence of the α1 chain in mouse adult tissues (Sasaki et al., 2002a,b). The distribution of laminin-121 in adult tissue may thus be much more extensive than previously thought.

In vitro analyses revealed that laminins are involved in a variety of biological functions. They self-assemble and form structural networks through the interactions of their N-terminal (LN) domains and with other extracellular matrix proteins. Laminins bind integrin receptors, α-dystroglycan and syndecans to mediate cell adhesion to the extracellular matrix and transmit signals to cells. Major cell-binding sites are located within the C-terminal laminin globular (LG) domains of the α-chains. However, the coiled-coil rod structure composed of α-, β- and γ-chains is essential for the integrin binding (Deutzmann et al., 1990). EHS-laminin was shown to promote neuronal survival and neurite outgrowth (Edgar et al., 1988). These activities are mainly mediated through interactions between laminins and integrins. However, different laminin isoforms show distinct cell-binding activities, they stimulate neurite outgrowth differently and for some isoforms this depends on neurotrophins (Plantman et al., 2008).

In order to characterize the properties of laminin-121 *in vitro*, we have recombinantly expressed it together with laminins-111, -211 and -221 and compared their integrin binding and neurite outgrowth activities *in vitro*.

2. Results

2.1. Production and purification of recombinant laminins

HEK 293 cells were first transfected with the mouse γ1 chain expression vector and the clone which expressed the highest amount of laminin γ1 chain was selected for the following transfections with either mouse laminin β1 or β2 chain expression vectors. Western blots of cell lysates and conditioned media from the transfected cells are shown in Fig. 1. Most of the intact laminin γ1 chain was found in the cell lysate, while a faint band around 200 kDa and a strong band at 66 kDa were detected in the conditioned media (Fig. 1A). The anti-γ1 chain antibody used was raised against mouse laminin γ1 VI/V fragment (Garbe et al., 2002): referred to as γ1 LN+ LEa domains according the current nomenclature of the laminin domains (Aumailley et al., 2005). The recombinant fragment consisting of γ1 LN+ LEa domain migrated as a 55 kDa band on SDS-PAGE under reducing conditions (Gersdorff et al., 2005) and the 66 kDa band seen on the western blot likely consisted of LN, LEa, L4 and parts of LEb domains. Like the γ1 chain, most of β1 and β2 chains were not secreted into the media but were detected in the cell lysates (Fig. 1B and C). These γ1β1 and γ1β2 transfected HEK293 cell clones were further transfected with either mouse laminin α1 or α2 chain expression vector and selected with puromycin (0.5 μg/ml). After triple transfection, the laminins were secreted into the media as shown previously (Yurchenco et al., 1997) and we observed that single α1 and α2 chains produced in excess were also secreted (data not shown).

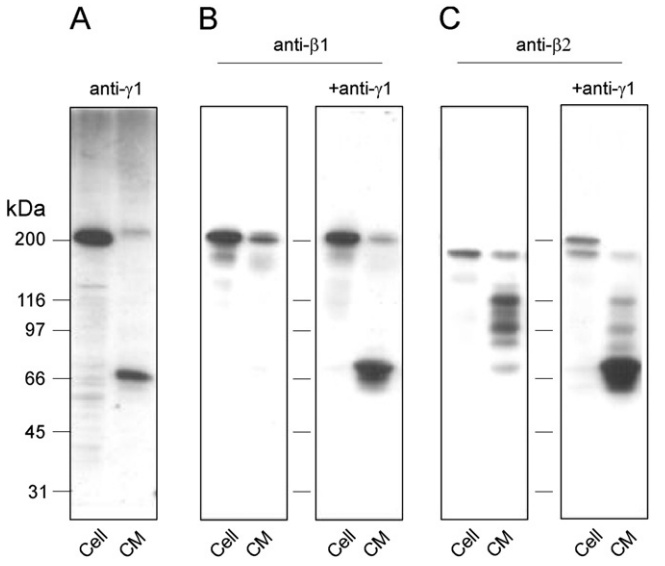

Fig. 1. Immunoblot analysis on the transfected HEK 293 cells. Cell lysates and conditioned media from transfected cells were separated on 3–12% acrylamide gels under reducing conditions and then transferred onto PVDF membranes for the immunodetection with anti-mouse laminin γ1, anti-laminin β1 and anti-laminin β2 chain antibodies. A; γ1-tansfected cells, B; γ1β1-transfected cells, C; γ1β2-transfected cells. Cell; cell lysate, CM; conditioned medium. For B and C, the blots were incubated with anti-β1 or anti-β2 (left blot) prior to the detection of the γ1 chain (right blot).

Serum-free conditioned media were collected and used for the purification. The estimated productions were 3–5 mg/l for laminin-111, 2–4 mg/l for laminin-121, 2–3 mg/l for laminin-211 and 1–2 mg/l for laminin-221. All these recombinant laminins were bound to Heparin-Sepharose 4B and were further purified with molecular sieve chromatography using a Superose 6 column.

2.2. Characterization of recombinant laminins

The purity of recombinant laminins is shown in Fig. 2. Under non-reducing conditions (Fig. 2A), all recombinant laminins were seen close to the top of the gel like EHS-laminin and these bands reacted with anti-α (α1 or α2), anti-β (β1 or β2) and anti-γ1 chain antibodies indicating that these laminins were produced as disulfide-linked heterotrimers (data not shown). Under reducing condition, two bands were seen for recombinant laminin-111. The 400 kDa band corresponds to the α1 chain and a 200 kDa band corresponds to the β1 and γ1 chains, but both bands migrated slightly faster than those of EHS-laminin suggesting that

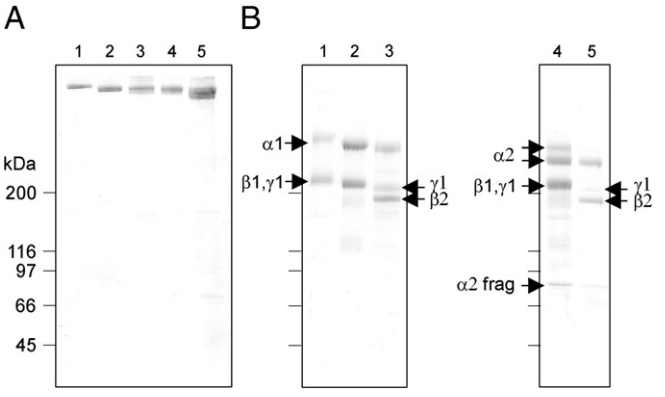

Fig. 2. SDS-gel electrophoresis of purified recombinant laminins. EHS-laminin (1), laminin-111 (2), laminin-121 (3), laminin-211 (4) and laminin-221 (5) were separated under non-reducing (A) and reducing conditions (B) and gels were stained with Coomassie Brilliant Blue R-250. The position of the individual laminin chains was independently confirmed by immunoblotting with chain-specific polyclonal antibodies.

EHS-laminin is more glycosylated than the recombinant one (Fig. 2B, lanes 1 and 2). Recombinant laminin-121 appeared as three bands (400 kDa, 200 kDa and 180 kDa), corresponding to the α1, γ1 and β2 chains respectively (Fig. 2B, lane 3). Four bands were seen for recombinant laminin-211 and -221 (Fig. 2B, lanes 4 and 5). The upper two in laminin-211 and a 300 kDa band in laminin-221 correspond to the α2 chain. The α2 chain was processed partially for laminin-211 and almost completely for laminin-221 and the resulting fragment, an 80 kDa band, was seen in both laminins under reducing condition. This band was not clearly seen with Coomassie Brilliant Blue staining under non-reducing condition (Fig. 2A, lanes 4 and 5) but it was definitely detected with the anti-α2 LG1-3 antibody (data not shown), indicating that this fragment is associated non-covalently.

Rotary shadowing electron microscopy revealed that these recombinant laminins have one long and three short arms (Fig. 3) as seen for EHS-laminin (Engel et al., 1981) and showed that all recombinant laminins were properly assembled.

The CD spectra of recombinant laminins were obtained at 4 °C (native state) and at 85 °C (heat-denatured state) and were very similar to that of EHS-laminin (data not shown) (Ott et al., 1982). Thermal transitions of EHS-laminin, recombinant laminin-111 and -121 are shown in Fig. 4. Melting curves for EHS-laminin and recombinant laminin-111 with their chain compositions α1β1γ1 closely match each other with $T_m = 62.5$ and 63.5 °C and a transition enthalpy $\Delta H_{VH} = 560$ and 770 kJ/mol, respectively. Error limits of T_m are ±1 °C but error limits for ΔH_{VH}-values determined from the slopes of the transition curves (Ott et al., 1982) are estimated to be ±10%. For recombinant laminin-121 (α1β2γ1) $T_m = 61$ °C is clearly smaller than for laminin-111 and $\Delta H_{VH} = 880$ kJ/mol is about 15% higher. The data indicate changes in thermodynamic values, which originate from the replacement of the β1 chain by the β2 chain. A summary of the melting curves of the laminins is shown in Table 1.

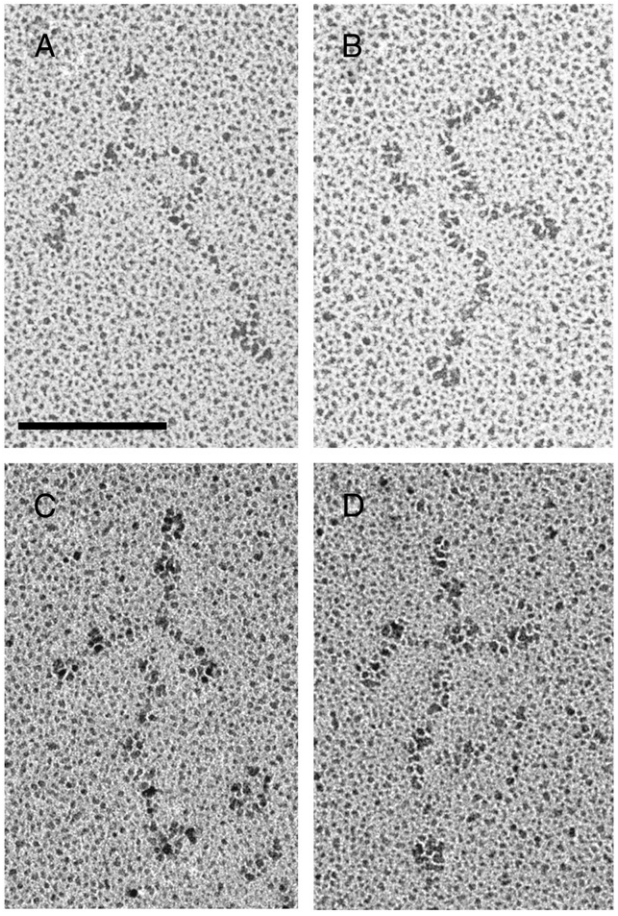

Fig. 3. Rotary shadowing electron microscopy of recombinant laminins. (A) laminin-111, (B) laminin-121, (C) laminin-211 and (D) laminin-221. Bar represents 100 nm. Each molecule shows typical cross-like structure of laminin having three short arms and one long arm.

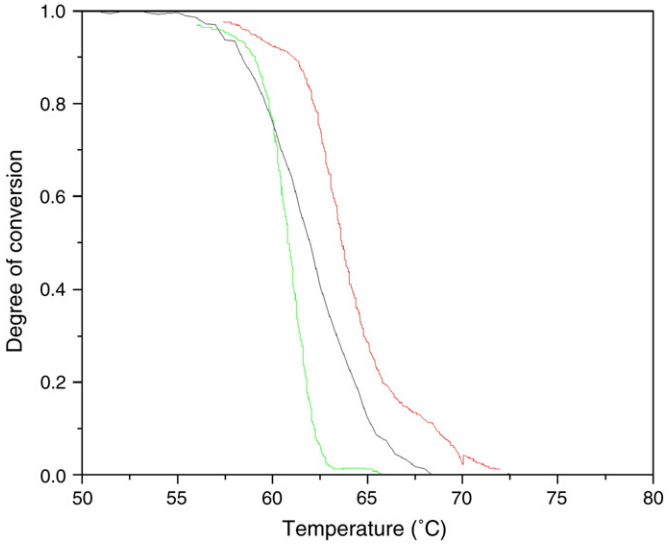

Fig. 4. Thermal unfolding of the coiled-coil domain of recombinant laminins-111 (red) and -121 (green) and EHS-laminin (black). The transition was followed by the change in mean molar ellipticity at 220 nm $[\Theta]_{220}$ and the degree of folding F was calculated by $F = ([\Theta]_{220} - [\Theta]_u)/([\Theta]_f - [\Theta]_u)$ where $[\Theta]_f$ and $[\Theta]_u$ are the ellipticities of the unfolded and folded state, respectively. Value of $[\Theta]_f$ and $[\Theta]_u$ showed small linear temperature dependencies, which were evaluated in the temperature range of 20 to 50 °C (folded state) and 70 to 85 °C (unfolded state) and subtracted in the evaluation of F.

2.3. Cell-binding activities of recombinant laminins

The human erythroleukemic K562 cells express only α5β1 integrin and therefore this cell line was chosen to express defined integrins (Delwel et al., 1993). In agreement with previous reports (Delwel et al., 1993) cell adhesion of α6 transfected K562 cells to different laminins showed that integrin α6 transfected K562 cells do not attach to the laminins tested without activation by the anti-β1 antibody TS 2/16 and only after activation these cells attached well to recombinant laminin-121 and laminin-3A32, and moderately to EHS-laminin, recombinant laminins-111, -211 and -221 (Fig. 5A). K562 cells transfected with α6 and β4 subunits express constitutively active α6β4 integrin and were shown to bind to EHS-laminin and laminin-3A32 without activation (Niessen et al., 1994). In our assay, α6β4 expressing K562 cells did not adhere to EHS-laminin or recombinant laminin-111 and bound weakly to recombinant laminin-121 and laminin-3A32 with low plateau levels (Fig. 5B). These data suggested that the K562 cells expressed low levels of α6β4 integrin and therefore significant attachment was seen only to laminins having higher affinities. Therefore the binding of laminin-121 to α6β4 was measured in a solid phase binding assay using recombinant α6β4 integrin. In this assay recombinant laminin-121 and laminin-3A32 bound well to α6β4 integrin while the binding of recombinant laminin-111 was weaker (Fig. 6).

Table 1
Summary of melting curves of recombinant laminins. The midpoint of the thermal unfolding transition (T_m) is shown together with the transition enthalpy change calculated using a two state transition.

Laminin	T_m (°C)	ΔH_{VH} (kJ/mol)
EHS	62.5 ± 1	560 ± 140
EHS (Ott et al.)	62 ± 1	720 ± 180
rec laminin-111	63.5 ± 1	770 ± 200
rec laminin-121	61 ± 1	880 ± 400
rec laminin-221	71.1 ± 1	600 ± 150

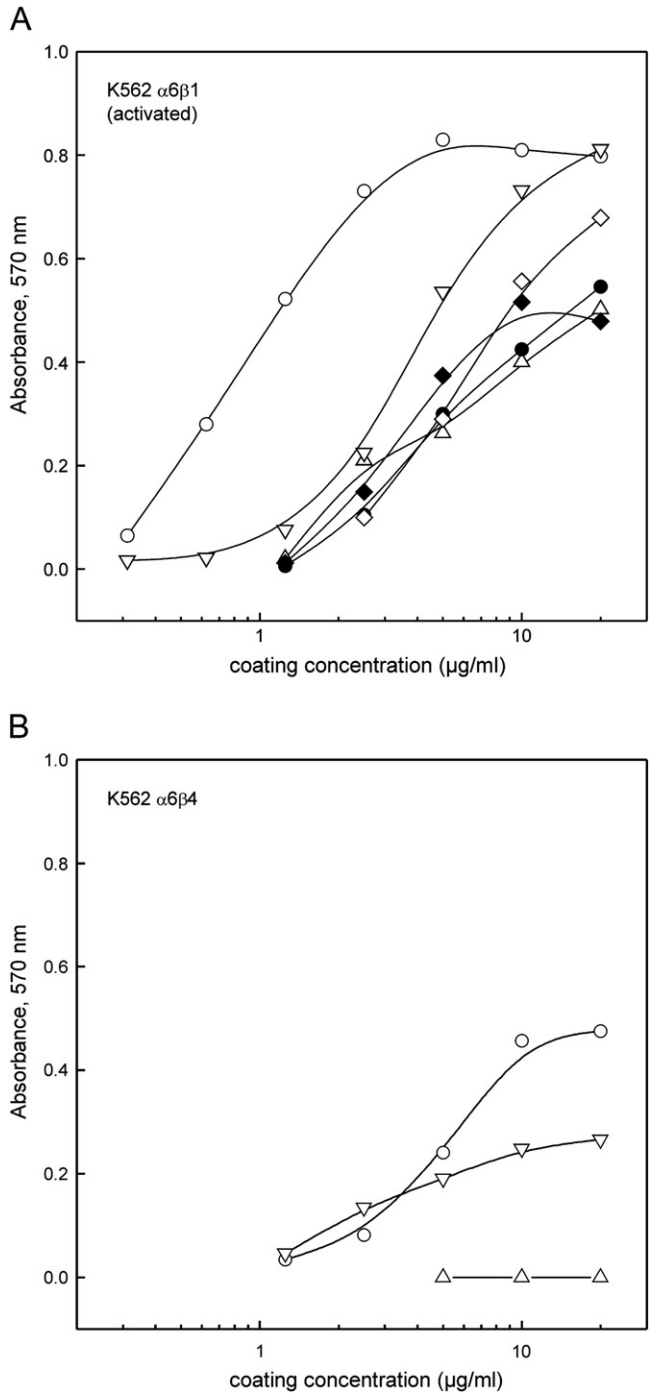

Fig. 5. Adhesion of α6 integrins transfected cells to recombinant laminins. α6 transfected K562 cells (K562/α6Aβ1) were stimulated with antibody TS 2/16 prior to the addition to 96 well plates coated with different laminins (A). K562/α6β4 cells were used without stimulation (B). EHS-laminin (△), laminin-3A32 (▽), recombinant laminin-111 (●), recombinant laminin-121 (○), recombinant laminin-211 (◆) and recombinant laminin-221 (◇). Cells were incubated at 37 °C for 60 min and the attached cells were stained with crystal violet. Representative examples of five experiments, each performed in duplicate, are shown.

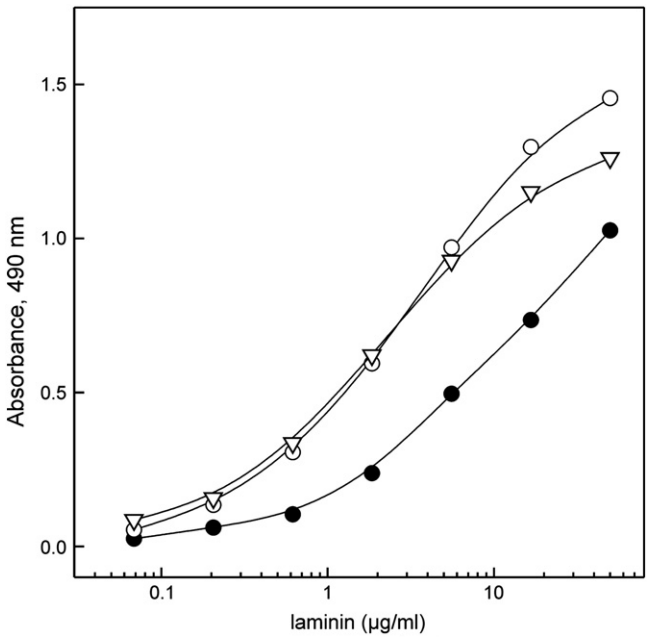

Fig. 6. Binding of recombinant laminins to immobilized α6β4 integrin. Recombinant soluble α6β4 integrin immobilized onto plastic wells and recombinant laminins-111 (●), -121 (○), and laminin-3A32 (▽) were used as soluble ligands. A representative of three experiments is shown.

Another receptor for laminins is α7β1 integrin and two extracellular splice variants were shown to differ in specificity and affinity to different laminin isoforms (von der Mark et al., 2002, 2007). It has been shown that the α7X2β1 variant binds strongly to laminin-111, while the α7X1β1 variant has a higher affinity to laminin-411 and laminin-511/-521. Here we tested adhesion of α7X2 transfected K562 cells to our recombinant laminins in the presence or absence of the β1 integrin-activating antibody TS2/16. In the absence of the β1 integrin-activating antibody, cells attached strongly to laminin-121, only weakly to laminin-111, but not to laminin-211 or -221 (Fig. 7A). After activating with TS2/16, cell attachment to all laminins tested was significantly enhanced (Fig. 7B). Overall, the data demonstrate that recombinant laminin-121 has the highest affinity to α6β1 and α7β1 integrins among the laminins tested.

2.4. Neurite outgrowth

To investigate the ability of the recombinant laminins to support neurite outgrowth, dissociated DRG neurons from adult mice were plated in 4-well culture dishes coated with the recombinant laminins and EHS-laminin. After culture overnight in the absence of growth factors, abundant neurite outgrowth visualized by labeling with βIII tubulin was observed on all laminins (Fig. 8) and strikingly greater than in control (untreated) wells. The proportions of neurons extending neurites, mean neurite lengths and also levels of significance of their differences compared to controls are summarized in Table 2. All recombinant laminins supported neurite outgrowth in a similar manner to EHS-laminin, however, the β2 chain containing laminins-121 and -221 appeared to be more effective than the β1 chain containing laminins-111 and -211. The proportion of neurons extending neurites, however, was highest on laminin-121 and significantly greater than on laminin-211 ($p<0.05$). Mean neurite lengths on laminin-221 and EHS-laminin were similar to laminin-121, and significantly higher than on laminin-111 and laminin-211.

3. Discussion

In this study, recombinant mouse laminin-121 together with mouse laminins-111, -211 and -221 were expressed as native proteins without tags and purified with conventional chromatography. HEK 293 cells were transfected with the expression plasmids for mouse laminin γ1, β1/β2 and α1/α2 chains consecutively. In the absence of the α chain, the majority of the intact γ1 and β1/β2 chains remained intracellular, although small amounts of the chains were secreted. Interestingly, the γ2 chain can be secreted as a single chain demonstrated by recombinant

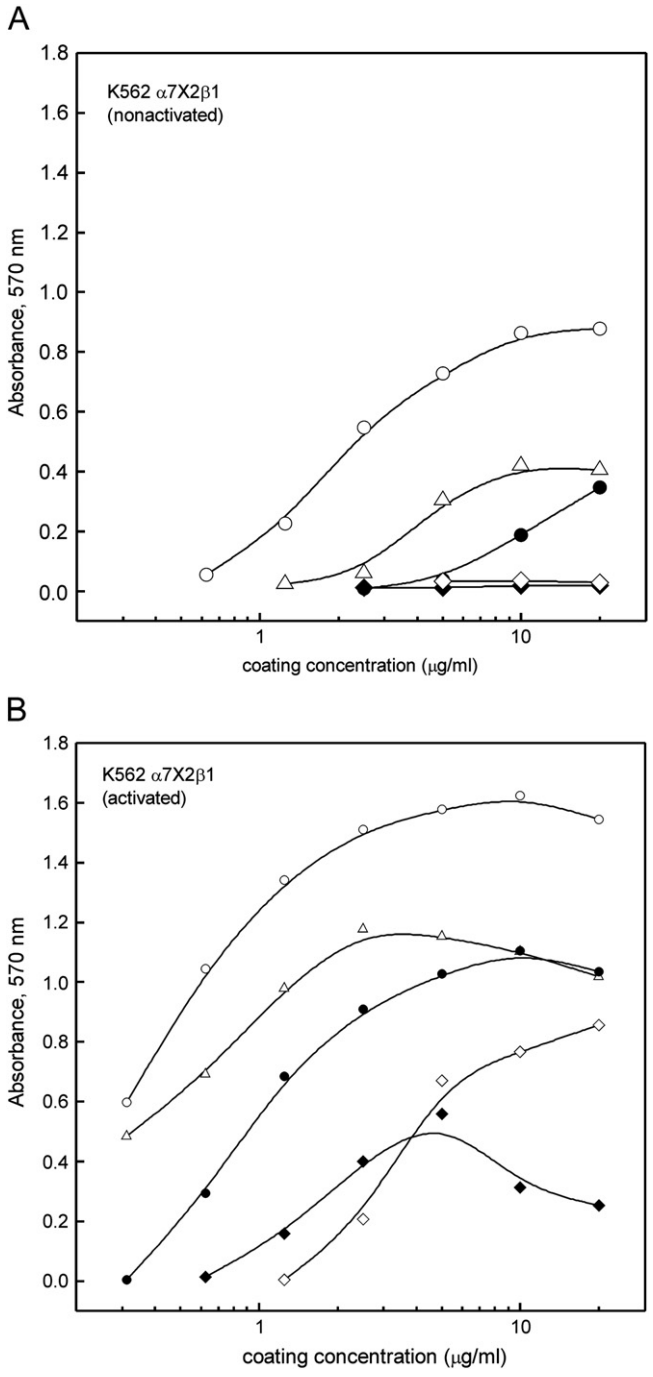

Fig. 7. Adhesion of the X2 variant of α7 integrin transfected K562 cells to recombinant laminins. A, cells were not stimulated with antibody TS 2/16. B, cells were stimulated with the antibody. EHS-laminin (△), recombinant laminin-111 (●), recombinant laminin-121 (○), recombinant laminin-211 (◆) and recombinant laminin-221 (◇). Representative examples of five experiments, each performed in duplicate, are shown.

expression (Kariya et al., 2002) and was found to be expressed in monomeric form by several malignant cancer cells (Koshikawa et al., 2008). Recombinant monomeric full-length γ2 chain could be purified; it was resistant against proteases compared to other laminin chains (Sasaki et al., unpublished). Cotransfection of either the α1 or α2 chain induced the secretion of the β and γ chains as heterotrimers as described previously (Yurchenco et al., 1997). Yurchenco et al. (1997) also demonstrated that the α1 chain was secreted in the absence of its partners, the β1 and γ1 chains, while the α2 chain was also secreted as a single chain when it was in excess of the β and γ chains. These monomeric α chains were separated from the heterotrimer by molecular sieve chromatography. All recombinant laminins expressed were properly folded as shown by rotary shadowing electron microscopy having cruciform structures typical for most of laminin isoforms.

The thermal stability of coiled-coil region in recombinant laminin-121 was similar to that of EHS-laminin and other recombinant laminins, showing only minor differences. We used at least two different preparations of each laminin for CD analyses and the data for each laminin were quite similar except for recombinant laminin-211, which showed a biphasic melting behavior with different ratios, indicating the presence of impurities or heterogeneity of the preparation. The α2 chain is processed in its LG3 domain by a furin-like protease and the resulting fragment is non-covalently associated to LG1-3 domains of the α2 chains (Talts and Timpl, 1999; Smirnov et al., 2002). The processing of recombinant laminin-211 was incomplete in contrast to laminin-221 and this heterogeneity may also affect its thermal stability, although further detailed investigation is needed. Lindblom et al. (1994) reported the CD analysis of laminins isolated from human placenta (mainly laminin-211) and from bovine heart (mixture of laminins-211 and -221) and these laminins showed a significantly higher thermal stability than EHS-laminin. The comparison of laminins-121 and -221 also indicated that laminins containing α2 chains have a higher T_m than α1 chain containing laminins. Furthermore the T_m of recombinant laminin-411 was only 59.5 ± 1 °C indicating that the α4 chain confers less stability to the coiled-coil region than the α1 chain (Sasaki et al., unpublished). Different stability of laminin isoforms may affect their susceptibility to proteases and this may be important during tissue regeneration.

The major laminin-binding receptors are α3β1, α6β1, α6β4 and α7β1 integrins (Hynes, 2002), each having distinct preferences for specific laminin isoforms. For example, laminin-111 binds to integrins α6β1, α6β4 and α7β1, especially to the α7X2β1 variant of α7 integrin with lower affinity to the α7X1β1 splice variant. Laminin-3A32 binds to α3β1, α6β1, α6β4 but not to α7β1 integrins (Delwel et al., 1994; von der Mark et al., 2002; Nishiuchi et al., 2006). Mapping studies using EHS-laminin fragments obtained with limited proteolysis revealed that the elastase-derived fragment E8 contains the major binding site for integrins (Aumailley et al., 1987; Goodman et al., 1987). Fragment E8 is composed of the C-terminal one-third coiled-coil region and LG1-3, and removal of the coiled-coil region abolished its integrin-binding indicating that the coiled-coil structure is essential for the binding (Deutzmann et al., 1990). The neurite-promoting activity is also localized in fragment E8 consistent with its central role in integrin binding (Edgar et al., 1988; Deutzmann et al., 1990). Here we have analyzed the activities of different laminin isoforms in supporting cell attachment using K562 cells stably expressing α6β1, α6β4 or α7X2β1 integrins and in promoting neurite outgrowth of dissociated adult DRG neurons on different laminin isoforms. Laminin-121 exhibited the highest adhesion for all cells tested and also bound to soluble α6β4 integrin similarly to laminin-3A32. Laminin-221 showed slightly higher cell-binding activity than laminin-211. However, the difference was not significant as reported previously (Brown et al., 1994). In summary, the β2 laminins (laminins-121 and -221) had higher affinity for integrins than the β1 laminins (laminins-111 and -211); this applied to both X1 and X2 splice variants of α6 or α7 integrins. These data stand in some contrast to recently published work claiming that β2 laminins bind more avidly to integrins bearing an X2 splice variant in the α chains, while X1 splice variants did not show any preference towards β2 laminins (Taniguchi et al., 2009). We have shown here, however, that laminin-121 had higher affinities to both α6X1β1 and α7X2β1 integrins, and there was no detectable preference for the β2 laminins between the X1 and X2 variants. Also in a previous study we have shown that both α7X1 and α7X2 splice variants bind with higher affinity to laminins-121 and -221 than laminins-111 and -211(von der

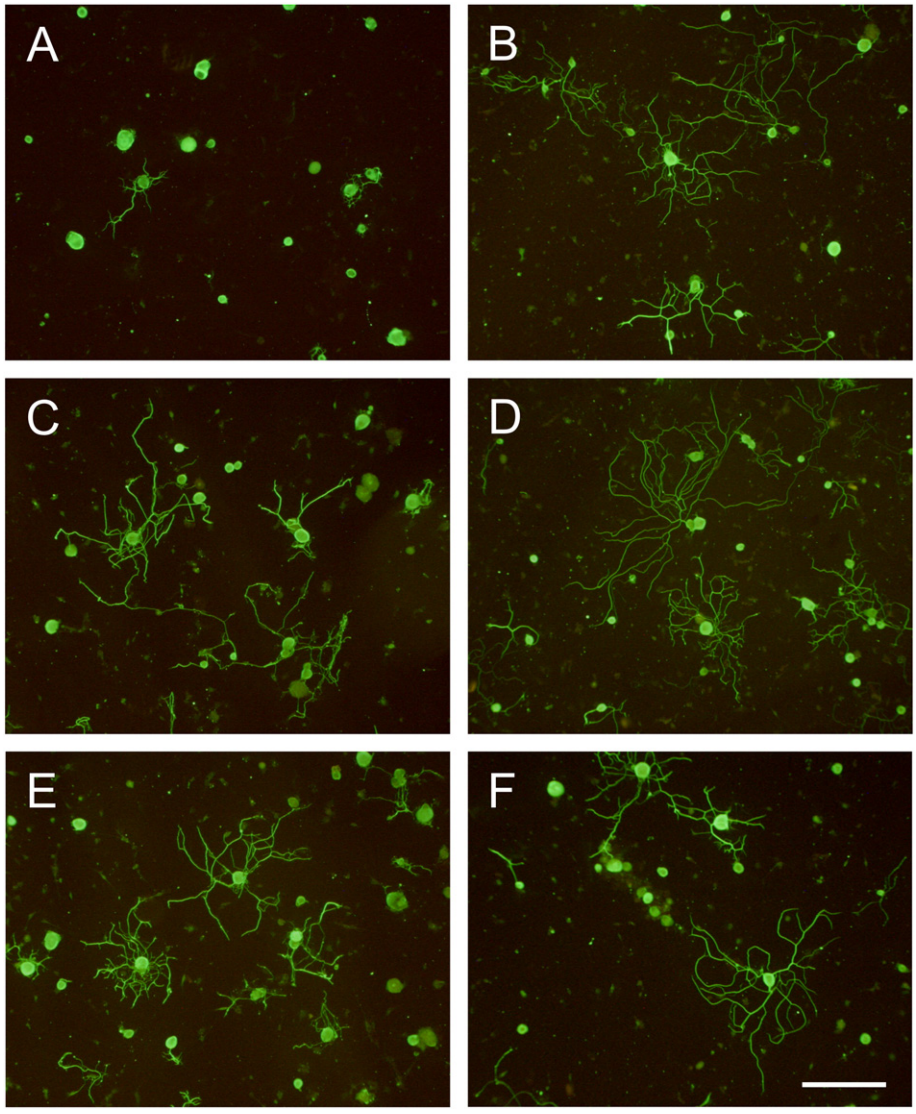

Fig. 8. Laminin isoforms differ in their ability to stimulate neurite outgrowth. Representative pictures of neurite outgrowth after overnight culture on different substrata were shown; uncoated (A), EHS-laminin (B), recombinant laminin-111 (C) recombinant laminin-121 (D), recombinant laminin-211 (E), recombinant laminin-221 (F). Bar represents 200 μm.

Mark et al., 2007). For the α7 integrin, both extracellular spliced variants X1 and X2 are differentially expressed during development and in adult tissues, but it is unclear, whether the α6X2 splice variant exists *in vivo*; the X1 variant of α6 integrin is ubiquitously expressed but the X1X2 mRNA is found only in a limited number of tissues and cell lines (Delwel et al., 1995). One possible explanation for the discrepancy between the data by Taniguchi et al. (2009) and ours may be the circumstance that in this study we have analyzed α1 and α2 laminins whose C-terminal LG domains are either not processed or still associate after being processed, while Taniguchi et al. (2009) used recombinant α5 laminins which were processed as well as various recombinant E8 fragments which have no LG4-5 domains. There are data, however, indicating that the LG4-5 domains may influence cell binding especially for the α1 and α2 laminins by interacting with the C-terminal domain of laminin β-chain, although the fragment E8 contains a minimal integrin-binding site. *In vivo*, the processed α5 chain was not found in some mouse newborn tissues (Yu and Talts, 2003), and the presence of its LG4-5 may influence its binding affinity for the integrins. Interestingly, different β-chains have a distinct effect on the processing of LG domains of laminins. Laminin-311 was secreted mainly as a non-processed form and this is quite different from laminin-332 which is often found as a processed form in cell culture and in tissues (Hirosaki et al., 2002). Our preliminary data on recombinant expression also indicate that the processed form of the α3 chain is prevalent in laminin-321 as compared to laminin-311 (Sasaki et al., unpublished). Formerly we have proposed a hypothetical model of laminin LG domains based on laminin α2 LG4-5 structure (Timpl et al., 2000). Accumulating data predict the close contact with the coiled-coil rod domain and LG domains, but the relative orientation of the rod and LG domain cannot be predicted in that model. It will be very interesting to know how the rod domain interacts with LG domains and how it modulates the integrin-binding and the processing of LG domains.

In the present study, we found that EHS-laminin and all recombinant laminins tested supported neurite outgrowth from adult mouse DRG neurons, even in the absence of added trophic factors.

Table 2
Summary of neurite outgrowth on different laminin isoforms. Mean lengths (in μm) of neurites and proportions of neurons extending neurites in parentheses on different laminins compared to controls (uncoated plastic) in 3 independent experiments. The levels of significance of increases compared to controls are shown in parentheses.

	Control	EHS Lm	rLm111	rLm121	rLm211	rLm221
Neurite lengths	132 ± 3	270 ± 16 (0.001)	213 ± 9 (0.001)	303 ± 31 (0.005)	201 ± 11 (0.005)	279 ± 4 (0.001)
% of neurons with neurites	14 ± 3	53 ± 7 (0.01)	36 ± 7 (0.05)	57 ± 7 (0.005)	30 ± 4 (0.05)	41 ± 6 (0.02)

Plantman et al. (2008) also found that EHS-laminin and recombinant laminin-511 supported spontaneous neurite outgrowth from DRG neurons, but on placental laminin-211 and recombinant laminin-411, abundant neurite outgrowth occurred only in the presence of NGF. The data on laminin-211 is inconsistent with ours as we observe neurite outgrowth also in the absence of NGF. A possible reason for the discrepancy may be different quality and properties of commercial laminin-211 used in the study by Plantman et al. (2008) versus our recombinant laminin-211. Wondimu et al. (2006) characterized commercial laminin preparations from human placenta and reported that different sources/batches of laminin-211 showed distinct reactivities with antibodies against laminin chains and also functional differences. On the other hand, several isoforms of recombinant laminins are now available (Kortesmaa et al., 2000; Doi et al., 2002; Kariya et al., 2002; Smirnov et al., 2002; Hayashi et al., 2006) and these laminins are quite useful for functional studies since it is difficult to isolate laminins from tissues without any contamination of other laminin isoforms.

In previous studies the laminin β2 chain has been found to act as a 'stop' signal for growing axons, due to presence of a C-terminal LRE sequence, and to be involved in organization of the neuromuscular junction and patterning of sensory innervation of the skin (Porter et al., 1995; Nishimune et al., 2004; Sann et al., 2008). However, we saw no evidence of inhibition of axonal growth on either of the β2 chain containing laminins. Consistent with our findings, Brandenberger et al. (1996) also observed that native laminin-221 promotes motor axon growth, and that inhibition of axonal growth by the LRE sequence depends on whether it is exposed or cryptic within the 3-dimensional helix of laminin. Moreover, the LRE sequence is conserved only among rodents but the corresponding sequence in human is LRG. It has not been demonstrated that the LRG sequence also acts as 'stop' signal.

The particular effectiveness of laminin-121 in supporting neurite outgrowth may be a consequence of its strong affinity for α7β1 integrins. α7β1 integrin is known to be important for axonal regeneration *in vivo* (Werner et al., 2000; Ekström et al., 2003) and *in vitro* studies have shown that it mediates neurite outgrowth on EHS-laminin (Gardiner et al., 2005) and also on laminin-211, but not on laminin-411 or -511 (Plantman et al., 2008). However, laminin-121 also showed strong affinity for α6β1 integrin and although this receptor mediates neurite outgrowth on laminins-411 and -511, but not on laminins-111 or -211 as shown by blocking antibodies against integrins (Plantman et al., 2008), its affinity for different laminin isoforms (Nishiuchi et al., 2006) does not appear to correlate with their ability to support neurite outgrowth. However, laminin-111 has not been shown to bind to α3β1 integrin and therefore it is unclear why the neurite outgrowth on laminin-111 was inhibited by anti-α3 integrin but not by anti-α6 integrin antibodies (Plantman et al., 2008). Further validation is required and the receptors mediating neurite outgrowth on laminins-121 and -211 remain to be determined.

The biological significance of the ability of laminin-121 in supporting neurite outgrowth of DRG neurons is uncertain since with the exception of the kidney, expression levels of the laminin α1 chain in most tissues of adult animals, including skeletal muscle and peripheral nerve, are very low (Sasaki et al., 2002a). However, some is present in skin (Sasaki et al., 2002a) where it is associated with the Pacinian corpuscles (Plantman et al., 2008) and may therefore be involved in their innervation.

The effectiveness of laminin-221 in supporting neurite outgrowth is interesting, since laminin chains α2, β1, β2 and γ1 are present in the endoneurium of peripheral nerves (Patton et al., 1997; Sasaki et al., 2002b) where they could associate to form laminins-211 and -221. As laminin-211 appears to be a poor substrate for neurite outgrowth (Plantman et al., 2008 and the present study) laminin-221 might therefore play a significant role in supporting axonal regeneration following peripheral nerve lesions.

4. Experimental methods

4.1. Expression constructs

A 0.74 kb fragment comprising bp 4259–5070 of mouse laminin γ1 chain cDNA was amplified with the primers 5′-CTAGCTCGAGCC-GATGCTG-3′ and 5′-GTCAGGTACCCTAGGGCTTCTCGATAG-3′ containing KpnI site after the stop codon. This PCR fragment was digested with XhoI and KpnI and cloned into the plasmid containing cDNA which encodes laminin γ1 L4 LE modules followed by LN module and the resulting plasmid contained bp 10–1668 and 4259–5070. A 3.6 kb fragment containing bp 847–4467 was obtained from the full-length γ1 cDNA (Sasaki and Yamada, 1987) with StuI and NheI and inserted into the above-mentioned plasmid to obtain full-length mouse γ1 chain cDNA. This plasmid was excised with EcoRV and KpnI and the fragment was inserted into pcDNA3.1/Hygro(−) (Invitrogen).

A 3.5 kb fragment containing bp1697–5538 was obtained from the retroviral expression plasmid for mouse laminin β1 chain (Yamada, unpublished) by using SalI and BamHI and cloned into pUC19 vector. From this plasmid, a SalI–KpnI fragment was excised and cloned into the pBluescript containing cDNA of bp 165–1803 of β1 chain. The full-length β1 chain cDNA was inserted into pcDNA3.1/Zeo(−) (Invitrogen) using NotI/KpnI sites.

In order to obtain mouse laminin β2 chain cDNA, the RNA from mouse embryonic endothelial cells (Hatzopoulos et al., 1998) was used for amplification by RT-PCR. Six PCR fragments of about 700–900 bp were amplified with the 5′-end and 3′-end fragments containing NheI and KpnI sites, respectively. The full-length cDNA of β2 chain was inserted into pcDNA3.1/Zeo(−) (Invitrogen) with NheI and KpnI.

The cDNA clones of mouse laminin α1 chain, La4 (bp1934–4354), La5 (bp3291–7769) and A18 (bp5945–9303) (Deutzmann unpublished) and the expression plasmids for LN+ 4LE (bp1–1575) modules (Garbe et al., 2002) and LG5 (bp 8749–9303) (Andac et al., 1999) were used to construct full-length cDNA. A 1.1 kb fragment containing bp 1461–2531 was amplified with the primers 5′-GTCAGGATCCATGCATAGAGCCGTG-3′ and 5′-GTCACCCGGGGCA-CATTGGTCAC-3′ using RNA from mouse embryonic endothelial cells as a template. The resulting full-length cDNA of laminin α1 chain was cloned into the episomal expression vector pCEP-Pu (Kohfeldt et al., 1998) via HindIII/NotI restriction sites.

The cDNA encoding LN+ 4LE modules (49–1593) of the α2 chain was cloned into Bluescript via XbaI/XhoI sites. The EcoRI/XhoI fragment was exchanged with the fragment obtained from the full-length cDNA clone of mouse laminin α2 chain (Bernier et al., 1995) and the resulting plasmid contains bp 49–2432. This cDNA was excised with XbaI and XhoI and cloned into the pCEP-Pu vector using NheI/XhoI sites. Another fragment comprising bp 2432–9506 was obtained from the full-length cDNA clone after digestion with XhoI and inserted into the above-mentioned plasmid to obtain the full-length cDNA of mouse laminin α2 chain in the pCEP-Pu vector.

All PCR fragments and correct ligations were verified by DNA sequencing.

4.2. Expression and purification of recombinant laminins

Recombinant mouse laminins were expressed in HEK 293 cells. At the first, cells were transfected with the expression plasmid for mouse laminin γ1 chain and selected with hygromycin B (Calbiochem) at 300 μg/ml. A clone highly expressing laminin γ1 chain was used for the second transfection with laminin β1 or β2 chain construct. The cells were selected using 300 μg/ml zeocin (Invitrogen). The cell lysates from zeocin-resistant clones were analyzed with antibodies against mouse laminin γ1, β1 and β2 chains and clones highly expressing both γ1 and β1/β2 chains chosen. Those clones were finally transfected with expression plasmids for either mouse laminin

α1 or α2 chains and the transfected cells selected with 0.5 μg/ml puromycin. All transfections were performed using the calcium-phosphate method (Chen and Okayama, 1987). The resistant cells were expanded and used for collecting serum-free conditioned media.

Conditioned media were passed over a Heparin-Sepharose 4B column equilibrated in 0.05 M Tris–HCl, pH 7.4 and laminin eluted with a linear 0–0.5 M NaCl gradient. All laminins were eluted at 0.2–0.4 M NaCl and were further purified on a Superose 6 column (HR16/50) equilibrated with 1 mM EDTA/0.3 M NaCl/0.05 M Tris–HCl, pH 7.4. Protein concentrations were determined on a Biotronik LC 3000 amino acid analyzer after hydrolysis with 6 M HCl (16 h, 110 °C).

4.3. Sources of proteins

Mouse laminin-111/nidogen-1 complex (EHS-laminin) was purified from Engelbreth-Holm-Swarm (EHS) tumor (Paulsson et al., 1987). Mouse laminin-3A32 was purified from serum-free conditioned media of Pam 212 cells. Briefly, proteins precipitated with 50% saturated ammonium sulfate were chromatographed on Superose 6 HR16/50 equilibrated with 0.3 M NaCl/0.05 M Tris–HCl, pH 7.4. The fractions containing laminin-3A32 were pooled and further purified with MonoQ HR5/5 and were used as controls.

4.4. Analytical methods

SDS-PAGE was performed using 3–12% gradient polyacrylamide gels under non-reducing and reducing conditions. For Western blotting, protein samples were run under reducing condition, blotted onto PVDF membranes (Millipore) and probed with rabbit antibodies against different domains of mouse laminin chains. They were anti-α1 VI/V, anti-α2 VI/V and anti-α2 LG4–5 (Sasaki et al., 2002a), anti-β1 IV, anti-β2 IV (Sasaki et al., 2002b) and anti-γ1 VI/V (Garbe et al., 2002). Confluent transfected cells grown in 10 cm dish were lysed in 1 ml of 0.05 M Tris–HCl, 0.01 M EDTA, 0.15 M NaCl, pH 7.4, containing 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride and 5 mM *N*-ethylmaleimide.

Electron microscopy of rotary shadowed proteins (Engel, 1994) and CD (Ott et al., 1982) followed standard protocols.

CD spectra were recorded on an Aviv 202 spectropolarimeter (AVIV Biomedical, Inc., Lakewood, NJ) using a Peltier thermostated cell holder and a 1-mm path length rectangular quartz cell (Starna Cells Inc., Atascadero, CA). Thermal transition curves were monitored at 220 nm and the temperature was increased by 10 K/h. The transition enthalpy was calculated from the slope of the transition curve using a two state model.

4.5. Binding analysis using recombinant α6β4 integrin

The construct design of soluble recombinant α6β4 is similar to that reported by Takagi et al. (2002) for αVβ3 ectodomain fragment containing C-terminal ACID/BASE peptides and a hexahistidine tag, except that extracellular portions of the α6 (residues 1–988) and β4 (residues 1–683) subunits were used. Soluble integrin was purified from culture supernatant of stably transfected 293EBNA cells using Ni-NTA agarose (QIAGEN) followed by anion-exchange (monoQ) and gel filtration (Superdex 200 HR) chromatographies.

Recombinant α6β4 immobilized onto plastic wells were incubated with various concentrations of laminins and bound laminins were detected with specific antisera either anti-laminin γ1 chain (for laminins-111 and -121) or anti-laminin γ2 chain (for laminin-3A32).

4.6. Cell adhesion assays

Dose–response profiles of cell attachment using substrates at different coating concentrations followed by a colorimetric assay were determined as described previously (Aumailley et al., 1989). The human erythroleukemic cell K562 stably expressing α6β1, α6β4 and α7X2β1 integrins (Delwel et al., 1993; Niessen et al., 1994; Sterk et al., 2002) were used for these analyses and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 U/ml). Cells were suspended in serum-free RPMI 1640 at 1×10^6/ml and preincubated with or without anti-β1 integrin antibody TS 2/16 (the conditioned media of TS 2/16 were added at 1:20) at 37 °C for 10 min. 100 μl of the cell suspension was added to wells coated with varying concentration of the laminins followed by incubation at 37 °C for 60 min. The attached cells were stained with 0.1% crystal violet for 30 min. The bound dye was solubilized in 0.1% Triton X-100 and the absorbance at 570 nm was measured.

4.7. Neurite outgrowth assay

Young adult female mice (Harlan) aged 4–5 weeks were killed by overdose of anaesthetic (euthanal). Lumbar dorsal root ganglia (DRGs) were removed by dissection and dissociated in 0.125% collagenase type III (Worthington) for 3 h at 37 °C, followed by trituration in 0.5 ml of RPMI medium containing 10% horse serum using a P1000 Gilson pipette. The dissociated cells were plated into 4-well dishes (Nunc), pre-coated with 20 μg/ml laminins in RPMI medium containing 100 units of penicillin, 100 μg streptomycin and 250 ng amphotericin B per ml at a density of approximately 1000 cells per well. Cultures were incubated for 18–20 h at 37 °C in an atmosphere of 5% CO_2 in a humidified incubator, followed by fixation for approximately 30 min with 3.6% paraformaldehyde in phosphate buffered saline (PBS) and subsequent immunocytology. After fixation and washing with PBS, cultures were blocked with 3% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) Triton X-100 in PBS for a further 30 min, prior to incubation at room temperature for 1 h with an antibody to βIII tubulin raised in rabbit (AbCam) followed by washing with PBS and labeling with Alexa 488-conjugated secondary antibody (1:300 dilution) raised in goat (Molecular Probes) for 30 min. Following a final wash in PBS, the cultures were mounted in Vectashield, (Vector Laboratories), coverslipped and viewed using an Eclipse TE200 fluorescence microscope and images captured directly using Nikon DXM1200F digital camera. Neurite outgrowth was quantified from 6 semi-random digital images per well by measuring the longest neurite of all isolated neurons (typically 4–6 per image) using a PC version of NIH Image (Scion Image). The proportions of all neurons in the images (typically 10–20 per image) extending neurites >1 cell diameter were also counted.

4.8. Statistical analysis

Results are expressed as means + S.E.M and *n* refers to the number of different animals used. The differences between means were evaluated by a Student's *t*-test and considered significant at $P<0.05$.

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