Syndecan-dependent binding of *Drosophila* hemocytes to laminin α3/5 chain LG4-5 modules: potential role in sessile hemocyte islets formation

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Received 7 July 2004; revised 16 August 2004; accepted 25 August 2004

Available online 15 September 2004

Edited by Masayuki Miyasaka

Abstract Heparin-column chromatography and elastase-digestion of medium from hemocyte Kc167 gave *Drosophila* laminin α3/5βγ trimer, α3/5LG2-3 and α3/5LG4-5 modules with eluting NaCl concentrations of 450, 280 and 450 mM, respectively. Kc167 cells bound dish surface with α3/5βγ trimer or α3/5LG4-5, but not with α3/5LG2-3 modules. Cell binding was counteracted by treating with heparin or heparan sulfate. RNA interference of *syndecan* in Kc167 cells impaired the binding, but that of d*ally* or *dally-like* did not. Green fluorescent protein-expressing hemocytes also bound surface with α3/5βγ trimer or α3/5LG4-5 module. Thus, syndecan-dependent binding of hemocytes to laminin may have a potential role in sessile hemocytes islets formation in T2-A8 segments of *Drosophila*.

Keywords: GFP; Hemolectin; Heparan sulfate; Heparin; LG module; RNA interference

1. Introduction

Sessile hemocytes are immobilized cluster of *Drosophila* blood cells, first described by Lanot et al. [1]. At least one third of blood cells are not in circulation, but attached to the inner surface of integument. We have visualized fluorescent hemocytes in *GAL4-hml; UAS-GFP* larvae [2,3]. Videotape recordings of these larvae can be viewed on our web site (http://www.agr.nagoya-u.ac.jp/~tagen/KitagawaG(Eng).html). These recordings confirm the observation by Lanot et al. [1] that sessile hemocyte islets are often located along the posterior region of the dorsal vessel, where hematopoiesis has been described in larger fly species [4,5]. The islets contain plasmatocytes and crystal cells, but not lamellocytes. Cells are linked through intricate interdigitations, but no desmosome nor septate junctions were observed between hemocytes [1]. Electron microscopy analysis of sessile islets provided no evidence for typical secretory cells and undifferentiated blast cells were rare [1]. It is thus not clear whether these islets result from the division of prohemocytes that were already present at the end of embryogenesis, or if they form later by the homing of circulating hemocytes to these locations during the larval stages. In addition to confirming the origin of sessile hemocyte islets, we identify biochemical molecules responsible for immobilization of hemocytes.

Kc167 is a *Drosophila* hemocyte-derived cell line that secretes large amounts of extracellular matrix proteins such as laminin, type IV collagen, glutactin and peroxidasin [6–8]. During culture, a proportion of Kc167 cells tends to attach weakly to the culture dishes. When attached, Kc167 cells often extend pseudopods which connect with sister cells just after division. After several days of culture, confluent cell populations spontaneously detach from the dish surface. Since a spinner culture gives almost the same growth rate, surface attachment is not necessary for the growth of Kc167 cells. Since cells become detached following addition of heparin to the culture, we postulate that laminin secreted by Kc167 cells is responsible for attachment to the plastic surface.

We purified *Drosophila* laminin α3/5βγ trimer by applying conditioned medium from Kc167 cells culture to heparin-columns. We also obtained α3/5 chain G domain fragments by running elastase-digested α3/5βγ trimer on a heparin column. Elution profile from heparin-columns by a NaCl gradient showed that α3/5 chain laminin G domain-like (LG)4-5 modules are responsible for the heparin-binding activity of the whole α3/5βγ trimer. By coating dishes with these proteins, we found that Kc167 cells bind to laminin at α3/5LG4-5 modules. Targeted knockdown of selected genes in Kc167 cells suggested that syndecan, a heparan sulfate proteoglycan (HSPG), may be the cell surface receptor for laminin. Thus, we postulate that syndecan-dependent binding of hemocytes is the underlying mechanism of sessile hemocyte islet formation.

2. Materials and methods

2.1. *Purification of* Drosophila *laminin α3/5βγ trimer*

Drosophila hemocyte-derived Kc167 cells were maintained under air at 25 °C in HyQ SFX-INSECT medium (Hyclone). 20 ml cultures were grown on Nunclone Surface dishes with diameter of 135-mm for laminin preparation. Conditioned medium was applied to a 1 ml heparin affinity column (HiTrap, Pharmacia) equilibrated with

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Abbreviations: BSA, bovine serum albumin; ds, double stranded; GFP, green fluorescent protein; HSPG, heparan sulfate proteoglycan; LG, laminin G-like domain; PBS, Dulbecco's phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; SDS, sodium dodecylsulfate

0014-5793/$22.00
doi:10.1016/j.febslet.2004.08.073

10 mM Tris–HCl (pH 7.4) containing 2 mM EDTA (buffer A). Elution was carried out by using a FPLC system (Pharmacia). The column was washed with 5 ml of buffer A, eluted with a 60 ml linear gradient of 0–600 mM NaCl in buffer A and 1 ml fractions were collected. Purity of laminin α3/5βγ trimer was monitored by sodium dodecylsulfate (SDS)-gel electrophoresis in 4% acrylamide gels under reducing conditions.

2.2. Elastase-digestion of laminin α3/5βγ trimer

Laminin α3/5βγ trimer was digested with porcine pancreas elastase (ES438, Elastin Products Co. Ltd. Mssooli). Incubation of α3/5βγ trimer at 160 μg/ml in buffer A with elastase at 5–10 μg/ml for 2 h at 37 °C was found to give the best yield. The digest was applied to a 1 ml Hi-Trap heparin column and purified as above. For SDS-gel electrophoresis, the fractions were separated in 12% acrylamide gels under reducing conditions. Separated peptides were transferred onto polyvinylidene difluoride membranes and stained with Coomassie brilliant blue G-250. Corresponding parts of the membrane were cut out and the peptides were sequenced from the N-terminus [9].

2.3. Cell binding assays

Binding of Kc167 cells to *Drosophila* laminin preparations was assayed in 96-well plates (Nunc). Plates were coated with various fragments diluted in Dulbecco's phosphate-buffered saline (PBS) overnight at 4 °C, rinsed with PBS and blocked with 1% bovine serum albumin (BSA) and washed with HyQ SFX-INSECT medium containing BSA. Cells in suspension culture were collected by centrifugation and suspended at 2×10^7 cells/ml and 100 μl aliquots were incubated in the wells for 2 h at 25 °C under air. After washing off unattached cells three times with PBS, attached cells were stained for 30 min with 0.4% (w/v) crystal violet in 50% (v/v) methanol. After washing three times with water, cells were dissolved in 50 μl of 0.1 M citrate in 50% ethanol and the absorbance at 595 nm was measured. To determine the effects of glycosaminoglycans and EDTA on the cell binding, the cells were preincubated with 10 μg/ml heparin, heparan sulfate, chondroitin sulfate A (All from Sigma) or 5 mM EDTA for 10 min and then subjected to the binding assay.

2.4. RNA interference of syndecan, dally and dally-like in Kc167 cells

RNA interference was carried out essentially as described [8,10]. Templates for the preparation of double strand (ds)RNA were PCR-derived fragments sandwiched by two T7 promoter sequences. The amplified cDNA fragments were: *syndecan* (nt 329–868, GenBank Accession No. U03282), and *dally* (nt 689–1528, U31985), *dally-like* (nt 1–1380, AF317090). Kc167 cells were plated in 6-well multidish plates (Nunc) and transfected with 6 μg of dsRNAs using Effectene Transfection Reagent (Qiagen). After three days of incubation, the transfected Kc167 cells were used for cell binding assay. To confirm the suppression of mRNA levels, RT-PCR was performed on mRNA preparations from the same Kc167 cells using sets of primers amplifying the sequences of *syndecan* (nt 929–1513), *dally* (nt 1649–2566), *dally-like* (nt 1441–2295) and *Hsc70-3* (nt 1506–1906, NM167308) as an internal control.

2.5. Binding assay of GFP-expressing Drosophila *hemocytes*

The fly line expressing GFP in hemocytes was made as described [3]. A chamber slide glass with 8-well (Nunc) was coated with BSA, *Drosophila* α3/5βγ trimer or α3/5 LG4-5 in PBS at the concentration of 100 μg/ml for overnight at 4 °C. After washing the chamber with PBS three times, the blocking with 1% BSA in PBS was done at 37 °C for 1 h. The chamber was washed with 0.1% BSA in PBS twice, loaded with hemolymph corresponding to 10 *hml-GAL4* × *UAS-GFP* larvae and incubated at 25 °C for 1.5 h. After careful washing with PBS three times, the micrograph of the wells was taken and the cell numbers were counted. Three independent binding assays were performed.

3. Results

3.1. High and low affinity heparin-binding sites in laminin α3/5βγ trimer

The affinity of *Drosophila* laminin α3/5βγ trimer to HSPG was used to purify it from conditioned medium of Kc167 cells using a heparin-column (Fig. 1A and B). A single protein peak was eluted from the column at NaCl gradient concentration of 450 mM (Fig. 1A). This peak contained laminin α3/5, β and γ chains as the major polypeptides as shown by SDS-gel electrophoresis under reducing conditions (Fig. 1B). This affinity of *Drosophila* laminin α3/5βγ trimer for the heparin-column was higher than that of mouse laminin-1 (α1β1γ1; eluted at 300 mM) and comparable with that of mouse laminin-8 (α4β1γ1; eluted at 470 mM) [12]. The yield of ca. 2.5 mg of purified protein from only 20-ml cultures indicates that laminin α3/5βγ trimer is a major protein secreted from the hemocyte cell line.

Mayer et al. reported that elastase and other proteases degraded *Drosophila* laminin to a small number of fragments with size ranging 40–300 kDa, and most of them bound to a heparin affinity column [11]. In Mayer's experiments, the fragments eluted at low salt concentration were derived from short arms of the α3/5βγ trimer, whereas a more strongly bound fragment of 50-kDa was from the long arm and contained the sequence of LG2-LG3 modules of α3/5 chain. By repeating essentially the same experiments (summarized in Fig. 1C and D), we could confirm that short arm fragments elute at low salt concentration. However, the fragment containing LG2-3 modules is not the fragment having the highest affinity. We obtained peaks I and II from the column at NaCl concentration of 280 and 450 mM, respectively (Fig. 1C). Single polypeptide in peak I had the size of 46 kDa and the N-terminal sequence of YGDDNDGGAR (Fig. 1D, left). This is the sequence adjacent to the beginning of LG2 module (Fig. 1E) [13]. The fractions of peak II gave doublet bands of ca. 46 kDa (Fig. 1D right) having the N-terminal sequence of LTTRPPAKL and NLPSDERCKLP, which are continued tandem sequences by 14 residues distant from the beginning of LG4 module (Fig. 1E) [13]. Our results clearly show that the fragment containing LG4 modules has higher affinity than that containing LG2 module. As depicted in Fig. 1E, the sizes of ca. 46 kDa for both fragments approximated to the calculated sizes of LG2-3 and LG4-5 module based on their sequence [13]. However, they have 4 and 2 predicted N-linked glycan addition sites, respectively [13]. If these sites were fully N-glycosylated, the fragments might lack the C-terminal sequence of LG3 or LG5 modules.

3.2. Kc167 cells bind to Drosophila α3/5βγ trimer at α3/5 chain LG4-5 modules

When Kc167 cells were incubated over a surface coated with either α3/5βγ trimer or α3/5 chain LG4-5 modules, they attached weakly and were resistant to gentle washing. Kc167 cells did not attach a surface coated with LG2-3 modules (Fig. 2A). LG4-5 module at a protein concentration 100 μg/ml attached two times more cells than α3/5βγ trimer. Considering the large difference between the sizes of the proteins (800 kDa for α3/5βγ trimer and 46 kDa for LG4-5 module), we tested the concentration-dependency of the attachment up to 1000 μg/ml of α3/5βγ trimer. As shown in Fig. 2B (upper panel), the α3/5βγ trimer at the maximal concentration over 500 μg/ml attached similar numbers of cells as LG4-5 module. When the data were replotted as molar ratios (lower panel of Fig. 2B), the two curves overlapped each other. This shows that attachment of Kc167 cells to the surface coated with α3/5βγ trimer can be explained mainly by the attachment to LG4-5

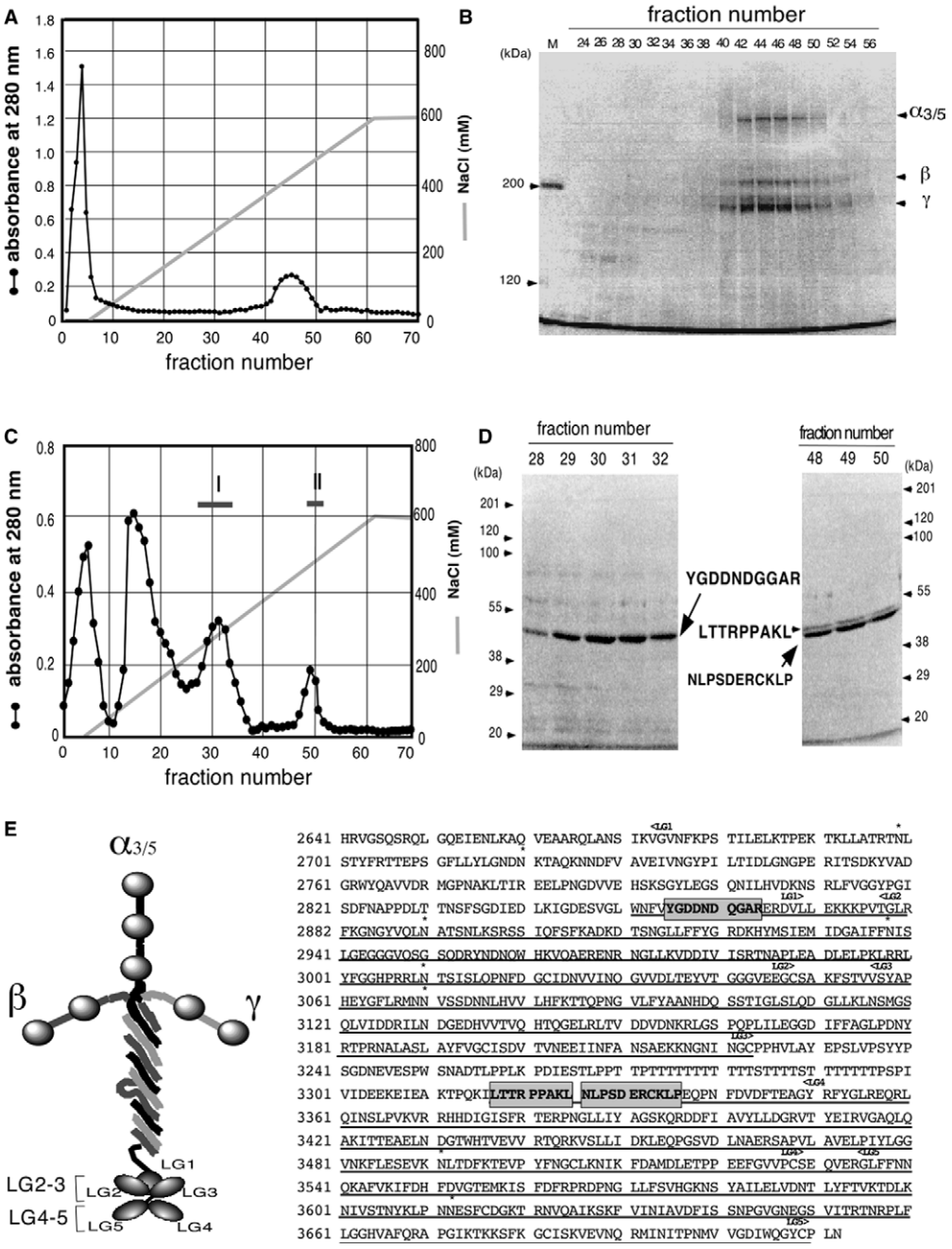

Fig. 1. High and low affinity heparin-binding sites in *Drosophila* laminin α3/5βγ trimer. Conditioned medium from Kc167 cell culture was applied to a heparin-column and eluted with linear gradient of NaCl from 0 to 600 mM. Protein elution was monitored by the absorbance at 280 nm (A). Purity of proteins in fractions was monitored by SDS-gel electrophoresis under reducing condition in a 4% acrylamide gel (B). Migration positions of laminin α3/5, β and γ chains are indicated at right. The digests of α3/5βγ trimer with elastase were applied to heparin column (C). After elution with linear gradient of NaCl from 0 to 600 mM, absorbance of fractions at 280 nm was determined. Purity and identity of digested polypeptides were studied by SDS-gel electrophoresis under reducing condition in a 12% acrylamide gel (D). N-terminal sequences of indicated polypeptides were determined. Migration positions of size marker proteins are indicated in margins. Outline structure of *Drosophila* laminin α3/5βγ trimer and amino acid sequence of *Drosophila* laminin α3/5 chain at the G domain [13] are shown in panel (E). The boundaries of LG1 through LG5 modules are indicated. Shadowed boxes indicate the sequences found in the elastase-digested fragments. Under lines indicate estimated regions contained in the fragments. Asterisks indicate the predicted N-linked glycan addition sites.

modules. Minor difference at the 0.1 μM range (Fig. 2B, lower panel) suggested contribution of other part of the α3/5βγ trimer molecule, but the contribution reduced the binding concentration to only a half. The results summarized in Fig. 2C show that attachment of the cells to LG4-5 module is due to their heparin-binding activity, since preliminary treatment of the cells with heparin or heparan sulfate diminished the attachment, but chondroitin sulfate did not. The lack of effect of EDTA suggested that integrins are not involved in the attachment (Fig. 2C).

Considering potential function of integrins in the binding of Kc167 cells to α3/5βγ trimer at the 0.1 μM range

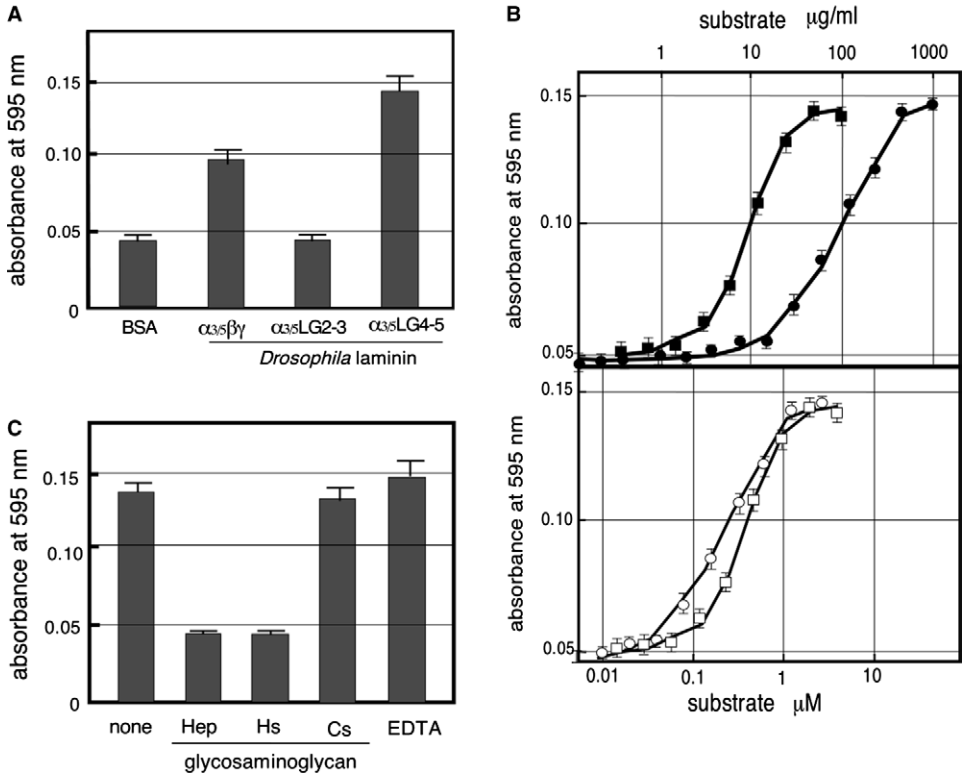

Fig. 2. Kc167 cells bind to *Drosophila* α3/5βγ trimer at α3/5 chain LG4-5 modules. 96-well multi-titer plates were coated with 100 μg/ml of α3/5βγ trimer, α3/5 LG2-3 or α3/5 LG4-5 and Kc 167 cells were allowed to attach at 25 °C for 2 hr. After washing off non-attaching cells, remaining cells were stained with crystal violet (A). Wells were coated with indicated concentration of α3/5βγ trimer (circles) or α3/5 LG4-5 modules (squares) (B). The attachment is plotted both on weight base (the upper panel) or on molar base (the lower panel). The cells were pretreated with none, heparin (Hep), heparan sulfate (Hs), chondroitin sulfate (Cs) or EDTA and incubated in the wells coated with 100 μg/ml of α3/5 LG4-5 modules. Averages for triplicate assay are shown with standard error bars.

(Fig. 2B, lower panel), we tested the effect of EDTA and observed the trend of suppressive effect with no statistic significance (not shown). Addition of LG2-3 modules to the α3/5βγ trimer coating at this concentration range showed no competitive effect (not shown). Addition of LG4-5 modules complicated the interpretation of data due to its own binding activity. Kc167 cells attached to α3/5βγ trimer and LG4-5 modules with similar morphological appearance.

3.3. *Kc167 cells bind to LG4-5 modules via syndecan but not glypicans (dally and dally-like proteins)*

An advantage of Kc167 cells is that expression of the gene of our interest can be knocked down just by the addition of dsRNA encoding a partial sequence of mRNA to the culture medium [8,10]. In the *Drosophila* genome, only three core proteins of HSPGs, namely syndecan, dally and dally-like proteins, have been identified. The latter two are homologous to mammalian glypicans. Therefore, it is easy to design experiments addressing the question which HSPG is important for the attachment of Kc167 cells to LG4-5. As shown in Fig. 3A, incubation of the cells with dsRNA encoding the mRNA sequence of *syndecan*, *dally* or *dally-like* caused specific reduction of the corresponding mRNA detected by RT-PCR. When the attachment of such cells to the surface coated with LG4-5 was tested, only the knockdown of *syndecan* resulted in a significant reduction of the attachment (Fig. 3B). Knockdown of *dally* and *dally-like* caused no decrease in the attachment.

3.4. *GFP expressing larval hemocytes bind to laminin α3/5 chain LG4-5 modules*

As described by Lanot et al. [1], a large population of sessile hemocyte is fixed to the inner surface of the integument. We visualized such cell clusters using a hemocyte-specific GFP expression system [3]. Fig. 4A and B are lateral view of the same larva to specify the dorsal/anterial localization of GFP-expressing hemocytes (Fig. 4B) by referring the position of denticle belts (asterisks in Fig. 4A). The dorsal view of a whole larva (Fig. 4C) shows that such localization pattern is repeated in T2, T3, and A1 through A8 segments.

To address the question what is the molecular base for such sessile hemocytes, we tested the binding of GFP-expressing hemocytes to the surface coated with *Drosophila* laminin. When hemolymph extracted from the larvae was overlaid, the surface coated with 100 μg/ml of α3/5βγ trimer showed a strong binding activity compared with the control surface (Fig. 5A(a) and (c)). The majority of bound cells expressed GFP, indicating that they were larval hemocytes (Fig. 5A(b) and (d). Interestingly, when coated with LG4-5 modules, stronger binding was observed (Fig. 5A(e) and (f)). The number of bound hemocytes was approximately twice of that for α3/5βγ trimer coating (Fig. 5B). This suggested that the GFP-expressing hemocytes bound to laminin fragments with a same concentration-dependency as for Kc167 cells (Fig. 2A and B).

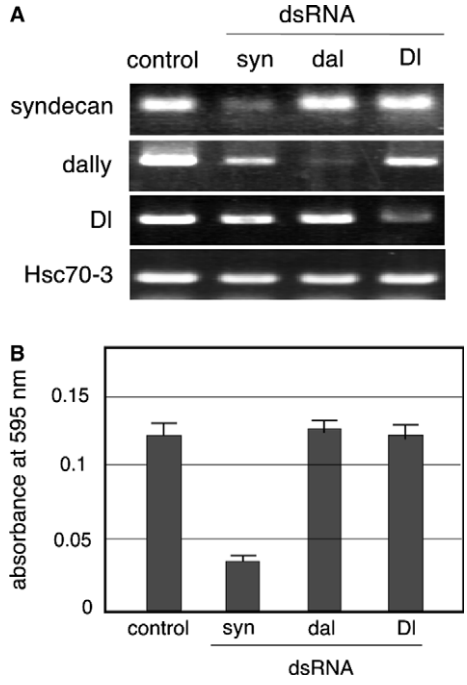

Fig. 3. Kc167 cells bind to LG4-5 modules *via* syndecan but not gypicans (dally and dally-like proteins). Targeted knockdown of *syndecan* (Syn), *dally* (Dal) and *dally-like* (Dl) by transfecting Kc167 cells with corresponding dsRNA was confirmed by detecting mRNAs by RT-PCR (A). Attachment of knockdown variants to wells coated with 100 μg/ml of α3/5 LG4-5 modules was tested. Averages for triplicate assays are shown with standard error bars.

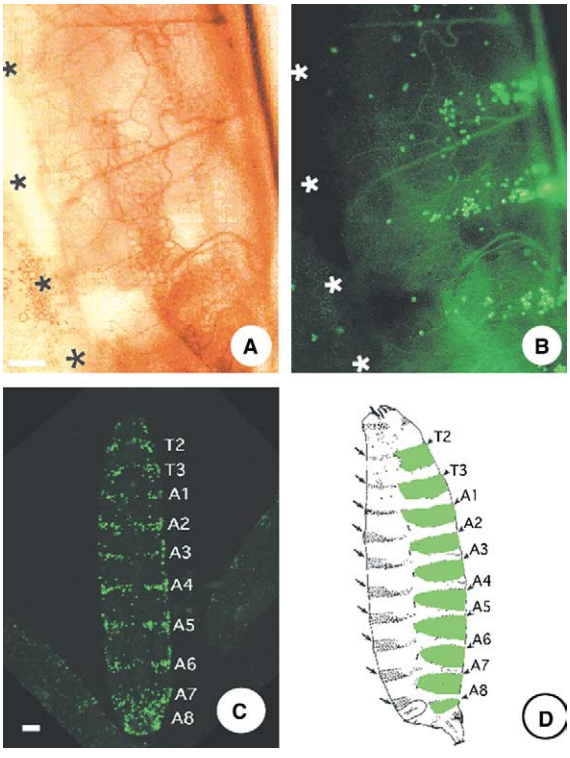

Fig. 4. Segmental localization of sessile hemocytes. *GFP*-expressing third (A and B) or second (C) instar larva under the control of *d-hml* promoter is visualized. Asterisks in (A) and (B) indicate denticle belts. Numbers (T2, T3, and A1 through A8 segments) in (C) are segment number. Lateral view (A and B) and dorsal view (C) are indicated. Segmental localization of hemocytes is sketched in (D).

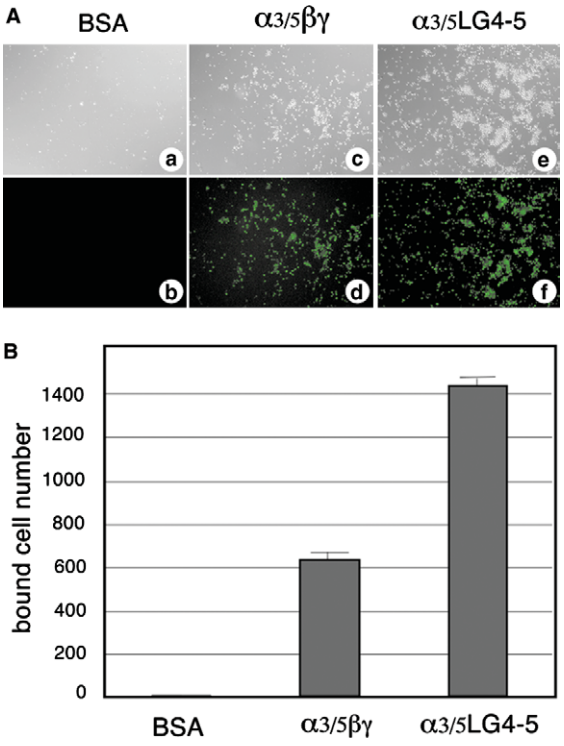

Fig. 5. GFP expressing larval hemocytes bind to laminin α3/5 chain LG4-5 modules. After coating with BSA (a and b), α3/5βγ trimer (c and d) or α3/5 LG4-5 modules (e and f), larval hemolymph containing hemocytes labeled with GFP was added, and the binding activity was assayed (A). Average number of bound cells from triplicate assays are shown with standard error bars (B).

4. Discussion

Localization of heparin-binding sites in the G domain of mammalian α chains has been completed. By limited proteolysis of recombinant mouse α1 G domain expressed in Sf9 insect cells, the relative affinity of LG modules to heparin was assigned as LG1 > LG2 = LG4 > LG5 > LG3, such that LG1 bound strongly and LG3 not at all [14]. Since LG1-3 modules are hidden within the intact structure of laminin long arm [15], however, the active heparin-binding site was localized exclusively in LG4 [16]. Studies on mouse α2 chain demonstrated a distinct binding of heparin to LG3 and LG5 modules [17]. Structural analysis of carbohydrate binding sites of α3 [18] and α5 chains [19] showed the LG4 modules to be the most active. We identified low and high affinity sites in the LG2-3 modules and the LG4 module, respectively [12]. Although studies of the heparin-binding activity of individual LG module are not completed, the higher affinity of LG4-5 modules than LG2-3 modules (Fig. 1) suggests that the localization of heparin-binding sites in the G domain of *Drosophila* laminin α3/5 chain is similar to that of mammalian α3, α4 and α5 chains rather than mammalian α1 and α2 chains. Similar analysis of another *Drosophila* laminin α chain, α1/2, might uncover the significance of differential expression and function of α1/2 and α3/5 chains during the fly development [20].

By targeted knockdown of either *syndecan*, *dally or dally-like*, we showed that syndecan is responsible for the attachment of Kc 167 cells to α3/5 LG4-5 modules. Not much is known about the glycan chains of these *Drosophila* cell surface

HSPGs, but the Hep II domain of fibronectin can bind heparan sulfate chains from mammalian glypican and syncedan with similar affinity [21]. It is difficult to understand how α3/5 LG4-5 modules could distinguish *Drosophila* syndecan and glypicans (dally and dally-like proteins) to result in the difference of cell attachment. The crucial difference between glypican and syndecan is that glypicans are anchored to the outer leaflet of the plasma membrane through phosphatidylinositol linkage [22,23] and may not have a cytoplasmic domain. A possible explanation for the dispensability of glypicans for cell attachment might be that even although glypican binds to α3/5 LG4-5 with similar affinity as syndecan, this binding cannot create the reorganization of the cytoskeleton as it is essential for cell adhesion.

The potential role of syndecan-dependent binding of hemocytes to laminin α3/5βγ trimer during formation of the sessile hemocyte islets is based on indirect evidence: GFP-expressing hemocytes in hemolymph attach to the surface coated with α3/5βγ trimer or α3/5 LG4-5 modules with similar concentration dependency as Kc 167 cells. Considering that hemocytes secrete a large amount of laminin α3/5βγ trimer, however, it is likely that inner surface of hemocoel and integument is coated with laminin. Hemocytes could attach to this laminin coating as observed during the culture of Kc 167 cells.

Acknowledgements: This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan awarded to Y.K. (15208034). A.G. is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. We thank Dr. Marie Meister, Dr. Jean-Marc Reichhart and Prof. David Gubb for critical proof reading of the manuscript.

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