Disulfide-bonding between *Drosophila* laminin β and γ chains is essential for α chain to form αβγ trimer

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Received 25 April 1997; revised version received 12 June 1997

Abstract Assembly of *Drosophila* laminin α, β and γ chains was analyzed by immunoprecipitation of the lysate from metabolically radiolabeled Kc 167 cells with chain-specific antibodies followed by two dimensional electrophoresis in which non-reducing and reducing SDS gel electrophoresis are combined. Precipitation of monomeric β (or γ) with anti-γ (or -β) antibody revealed that β and γ form stable dimer before they are disulfide-bonded to each other. In contrast, α associates with neither monomeric β, monomeric γ nor βγ dimer without disulfide-bonding but only with disulfide-bonded βγ dimer to form αβγ trimers. These results thus demonstrated that the interchain disulfide-boding between β and γ is essential for α to form αβγ trimer. We also found that the αβγ trimer can be secreted with α chain either disulfide-bonded or not bonded to the disulfide-bonded βγ dimer.

Key words: Basement membrane; Disulfide-bonding; *Drosophila*; Laminin

1. Introduction

Mammalian laminins constitute a family of basement membrane glycoproteins having strong effects on proliferation, migration and differentiation of various types of cells [1–4]. The best studied mouse Engelbreth-Holm-Swarm (EHS) laminin (laminin-1) is composed of α1, β1 and γ1 chain, assembled and disulfide-bonded in a cross shaped structure with three short arms and one rod-like long arm [5–7]. Structural analysis [8] and molecular cloning [9–11] have identified the long arm to be the site of chain assembly. It has many repeats of a heptad motif in which hydrophobic residues are at the first and fourth positions and charged residues are at the fifth and seventh positions [12]. They form a hydrophobic surface along the α-helix with two charged edges at both sides. Interchain hydrophobic interactions at this surface drive chain assembly and ionic interactions at the edges determine chain selectivity. In addition, β1 and γ1 are disulfide-bonded to each other at N- and C-termini of the long arm while α1 is disulfide-bonded to β1 and γ1 only at N-termini of the long arm. Our analysis on various mammalian cell lines [13–15] showed that β1 and γ1 first form disulfide-bonded β1γ1 dimer and then α1 (or its homologue) is disulfide-bonded to β1γ1 dimer to form the trimer. Due to the lack of chain-specific antibodies, however, the role of interchain disulfide-bonding in the process of chain assembly was not clear.

Drosophila laminin was first isolated by Fessler et al. and characterized to have α (400 kDa), β (220 kDa) and γ (200 kDa) chains [16]. The cDNA clones for α [17], β [18,19] and γ [20] have been isolated, *Drosophila* α has the domain organization similar to mammalian α5 with the sequence homology of 35% [21]. *Drosophila* β and γ have 41 and 30% sequence homology to mouse β1 and γ1, respectively [19]. *Drosophila* laminin has the conserved domains found in mammalian laminins such as epidermal growth factor (EGF)-like repeats and globular domains in the short arms and the heptad repeats to form α-helix coiled-coil structure in the long arm.

In this study, we prepared chain-specific polyclonal antibodies directed against *Drosophila* α, β and γ. Analysis with the antibodies revealed that β and γ form stable dimer before they are disulfide-bonded to each other. In contrast, α associates with neither monomeric β, monomeric γ nor βγ dimer without disulfide-bonding but only with disulfide-bonded βγ dimer to form αβγ trimers. These results demonstrated that the interchain disulfide-bonding between β and γ is essential for αβγ trimer formation.

2. Materials and methods

2.1. SDS-gel electrophoresis

SDS gel electrophoresis was carried out as described [13–15]. For one dimensional electrophoresis, 4% (w/v) polyacrylamide gel was used. In two dimensional electrophoresis, 4% gels were used for the first dimension under non-reducing condition and for the second dimension under reducing condition.

2.2. Preparation of anti-laminin antibodies

Conditioned medium of Kc 167 cell [22] cultured with HyQ CCM3 (HyClone Lab. Inc.) serum-free medium at 25°C was concentrated by ultrafiltration (Amicon CentriPlus 30) and the extracellular matrix proteins were partially separated by velocity sedimentation as described by Fessler et al. [23]. For this, 24 ml of a 7–30% sucrose gradient in a Hitachi SRP28SA rotor at 143000×*g* for 23 h was used and 1 ml fractions were collected for SDS gel electrophoresis under reducing condition (Fig. 1A). CBB (Coomassie brilliant blue) staining of the gel showed the same separation pattern as reported [23] except for the absence of papilin, which may be trapped to the filter for concentration. Co-sedimentation of three bands at the velocity consistent with the laminin trimer and their relative migration in SDS gel electrophoresis allowed us to assign the bands corresponding to the α, β and γ chains. For the preparation of antibodies, the conditioned medium was concentrated by trichloroacetic acid precipitation and separated by SDS gel electrophoresis (Fig. 1B), the gel pieces corresponding to the migration position of α, β, and γ were cut out, and used for immunization of rabbits. Anti-laminin α antibody was purified as described by Sambrook et al. [24]. Briefly, the medium was separated by SDS gel electrophoresis and blotted to Hybond ECL (enhanced chemiluminescence) membrane (Amersham). The antibody specifically bound to 400-kDa band was eluted with 0.1 M glycine-

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Abbreviations: CBB, Coomassie brilliant blue; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EHS, Engelbreth-Holm-Swarm; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; RIPA, radio-immunoprecipitation assay; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecylsulfate

PII S0014-5793(97)00780-1

HCl, pH 2.5 and the eluate was immediately neutralized by adding 1/20 volume of 1 M Tris and 1/10 volume of 10× phosphate-buffered saline (PBS). The anti-β and -γ antibodies were also affinity purified but they gave essentially the same results as the original antisera used in this study.

2.3. Immunoblotting

The medium after 22 h culture of Kc 167 cells or extracts from wild type *Drosophila* (Oregon R) at stage 17 embryos were separated by SDS gel electrophoresis and blotted to Hybond ECL membrane. The membrane was blocked with 5% (v/v) skim milk in PBS containing 0.1% (v/v) Tween 20 and reacted with the chain-specific antibodies. As a secondary antibody, a horse radish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) was used with dilution of 1:1000. Development was done with ECL Western blotting detection reagents (Amersham).

2.4. Cell labeling with [^{35}S]methionine and immunoprecipitation

Confluent Kc 167 cells were dispersed and transferred to 60 mm culture dishes (Nunc). After attachment of cells to dishes, the medium was replaced with the methionine-free M3 medium (Sigma) containing 0.25 mCi/ml [^{35}S]Pro-mix (Amersham) and incubated for 22 h at 25°C. Cells were lysed with radio-immunoprecipitation assay (RIPA) buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM EDTA, 0.25 mM phenylmethylsulfonylfluoride (PMSF), 1 mM *N*-ethylmaleimide (NEM), 0.3% (v/v) Nonidet P-40, 0.05% (v/v) Triton X-100, 0.3% (w/v) sodium deoxycholate, 0.1% (w/v) bovine serum albumin and 0.1% (w/v) SDS. The radiolabeled medium was adjusted to 1× RIPA buffer. The detergents in RIPA buffer were added at maximum concentrations to reduce non-specific precipitates while preserving the quantitative immunoprecipitation of laminin chains. After centrifugation at 12000×*g* for 5 min, aliquots of radiolabeled cell lysate and medium were incubated with the chain-specific antibodies for 16 h at 4°C followed by the incubation with 50 μl of 10% (w/v) Protein A-Sepharose CL-4B (Pharmacia) for 2 h at 25°C. The beads were washed three times with 500 μl of RIPA buffer and resuspended in an SDS sample buffer [15]. After boiling for 5 min, the samples were separated by two dimensional SDS-gel electrophoresis. After fixation with a solution containing 10% (v/v) trichloroacetic acid, 30% (v/v) methanol and 10% (v/v) acetic acid for 30 min, the gel was dried and analyzed with a Fuji Film BAS 2000 Image Analyzer.

3. Results

3.1. Chain-specific antibodies against Drosophila laminin α, β and γ chains

When the medium from Kc 167 culture was separated by SDS gel electrophoresis under reducing condition, laminin α, β and γ were detected among major proteins stained with CBB (Fig. 1A and B). Assignment of the bands corresponding to the α, β and γ chains was confirmed by co-sedimentation of the three bands in sucrose velocity sedimentation (Fig. 1A) [23] and their relative migration distance in SDS gel electrophoresis. We prepared chain-specific polyclonal antibodies directed against α, β or γ by immunizing rabbits with grounded gel piece containing each protein. Resulting antibodies gave strong signals at the migration positions of α, β and γ in an immunoblot of the medium separated by SDS gel electrophoresis under reducing condition (Fig. 1C, D and E, lanes under 'Kc'), showing the immunochemical specificity. When the extract from stage 17 *Drosophila* embryos was immunoblotted, weak bands with various sizes were detected in addition to the main bands of intact α, β and γ (Fig. 1C, D and E, lanes under 'emb'). The anti-α antibody detected bands migrating faster than intact α, suggesting that α is subjected to a processing. In contrast, anti-β and -γ antibodies detected bands migrating slower than intact β and γ (Fig. 1D and E, lanes under 'emb'). This may indicate that a part of β and γ is cross-linked to some components in the embryos through covalent bonding resistant to the reduction with 2-mercaptoethanol.

3.2. Assembly of Drosophila laminin in Kc 167 cells

To study the assembly of laminin in Kc 167 cells, they were metabolically labeled with [^{35}S]methionine/cysteine for 22 h and the radiolabeled cell lysate and medium were immunoprecipitated by chain-specific antibodies. The immunoprecipitates were separated by two dimensional SDS gel electrophoresis in which non-reducing electrophoresis in the first dimension (left to right) was followed by reducing electrophoresis in the second dimension (top to bottom). In this electrophoresis, monomeric proteins come to the diagonal while proteins disulfide-bonded to each other migrate to the area below the diagonal and form a vertical line for each disulfide-bonded complex.

When radiolabeled medium was immunoprecipitated either with anti-α, -β or -γ antibody, disulfide-bonded αβγ trimer, disulfide-bonded βγ dimer and α monomer were commonly precipitated in addition to a series of disulfide-bonded trimers containing β, γ and processed fragments of α (Fig. 2A, C and E). We will discuss these processed α fragments in the next section. Distinct from mammalian cells, Kc 167 cells secreted monomeric α (and its processed fragments) to the medium together with disulfide-bonded βγ dimer. In our previous studies on mammalian laminins [13–15], laminin chains left the endoplasmic reticulum for secretion only after the assembly into disulfide-bonded trimers. Neither monomeric chains nor disulfide-bonded βγ dimer was secreted. The results in Fig. 2 apparently show that such selection mechanism is not functioning in *Drosophila* cells. However, since α (and its processed fragments) is precipitated either with anti-β or -γ antibody (Fig. 2C and E) and disulfide-bonded βγ dimer is precipitated with anti-α antibody (Fig. 2A), it is clear that *Drosophila* α forms a stable αβγ trimer with disulfide-bonded βγ dimer. It indicates that *Drosophila* α is secreted after forming αβγ trimer even if it is not disulfide-bonded to disulfide-bonded βγ dimer. Considering the fact that stronger signal of monomeric α (and its processing product of 140 kDa) was found with anti-α antibody than with anti-β and -γ antibodies, however, there remained a possibility that small part of α chain is secreted as homotrimer suggested by Takagi et al. [25]. On the other hand, absence of monomeric β and γ in

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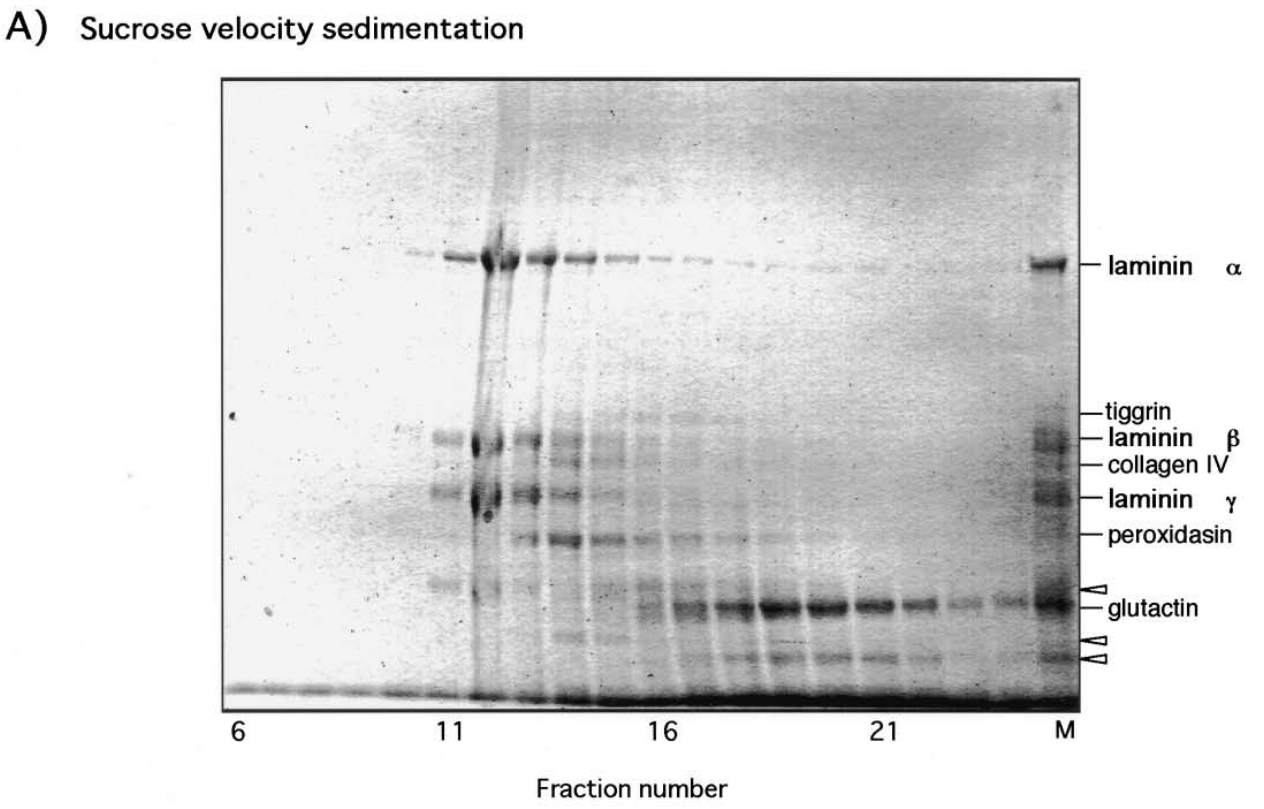
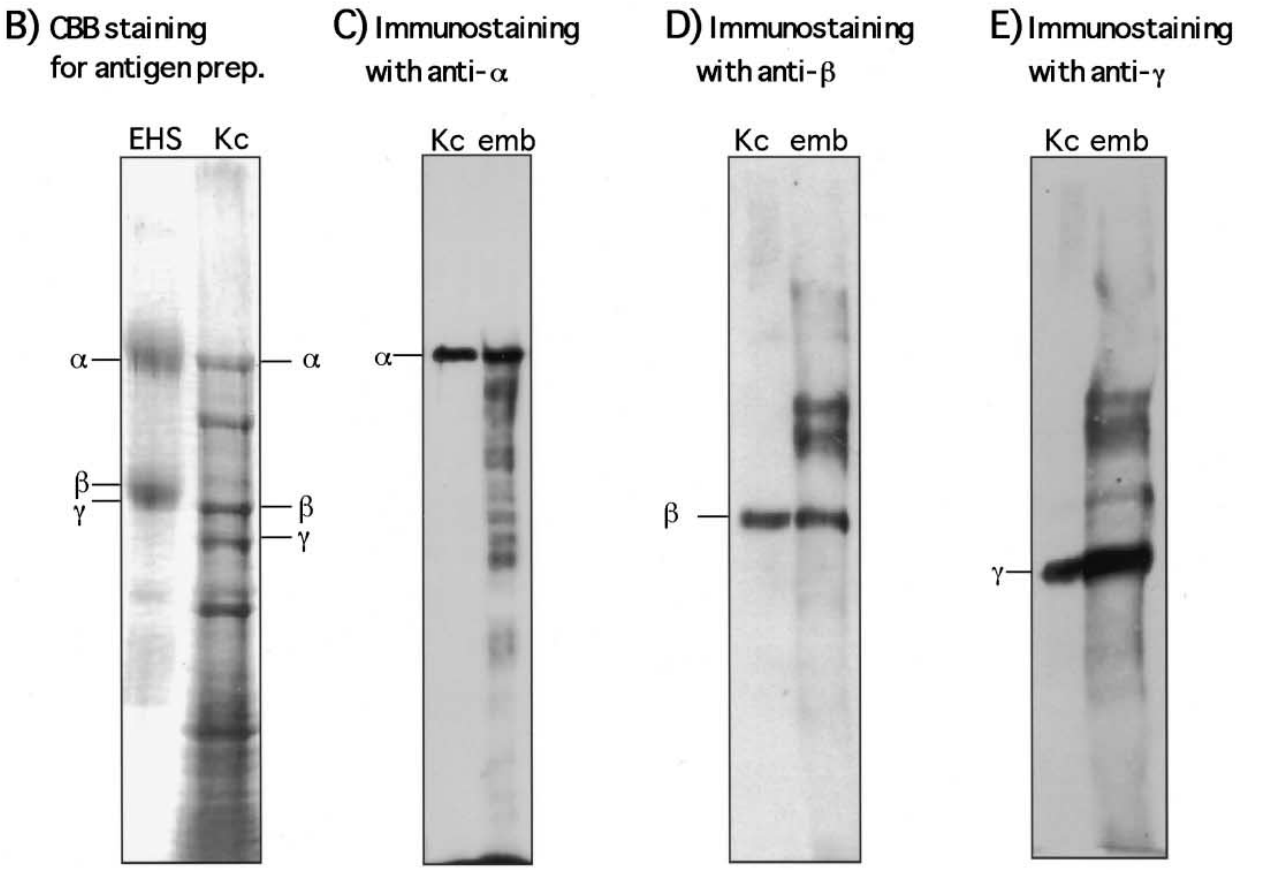
Fig. 1. Chain-specific antibodies directed against *Drosophila* α, β and γ. Panel A: The conditioned medium of Kc167 cells was separated by velocity sedimentation in a 7–30% sucrose gradient of 24 ml and fractionated into 1-ml aliquots (fraction 1 to be the bottom). CBB staining of the reduced SDS gel electrophoresis of the fractions is shown. The original conditioned medium was separated in the lane M. Assignment of each band is indicated at the right margin. Open arrowheads indicate unknown proteins. Panel B: Mouse EHS tumor laminin (EHS) and the conditioned medium of Kc 167 cell culture (Kc) after concentration by trichloroacetic acid precipitation were separated by 4% SDS-polyacrylamide gel under reducing conditions and stained with CBB. Panels C, D and E: 30 μl of the medium from Kc 167 cell culture for 22 h (Kc) and the extract from 150 embryos of wild type *Drosophila* at stage 17 (emb) were separated under reducing condition and immunoblotted with the affinity purified anti-α antibody (C), anti-β antiserum (D) or anti-γ antiserum (E). Migration positions of α, β, and γ chains are indicated.


A) Sucrose velocity sedimentation
laminin α
tiggrin
laminin β
collagen IV
laminin γ
peroxidasin
glutactin
6
11
16
21
M
Fraction number


B) CBB staining for antigen prep.
EHS
Kc
α
β
γ
C) Immunostaining with anti-α
Kc emb
α
D) Immunostaining with anti-β
Kc emb
β
E) Immunostaining with anti-γ
Kc emb
γ

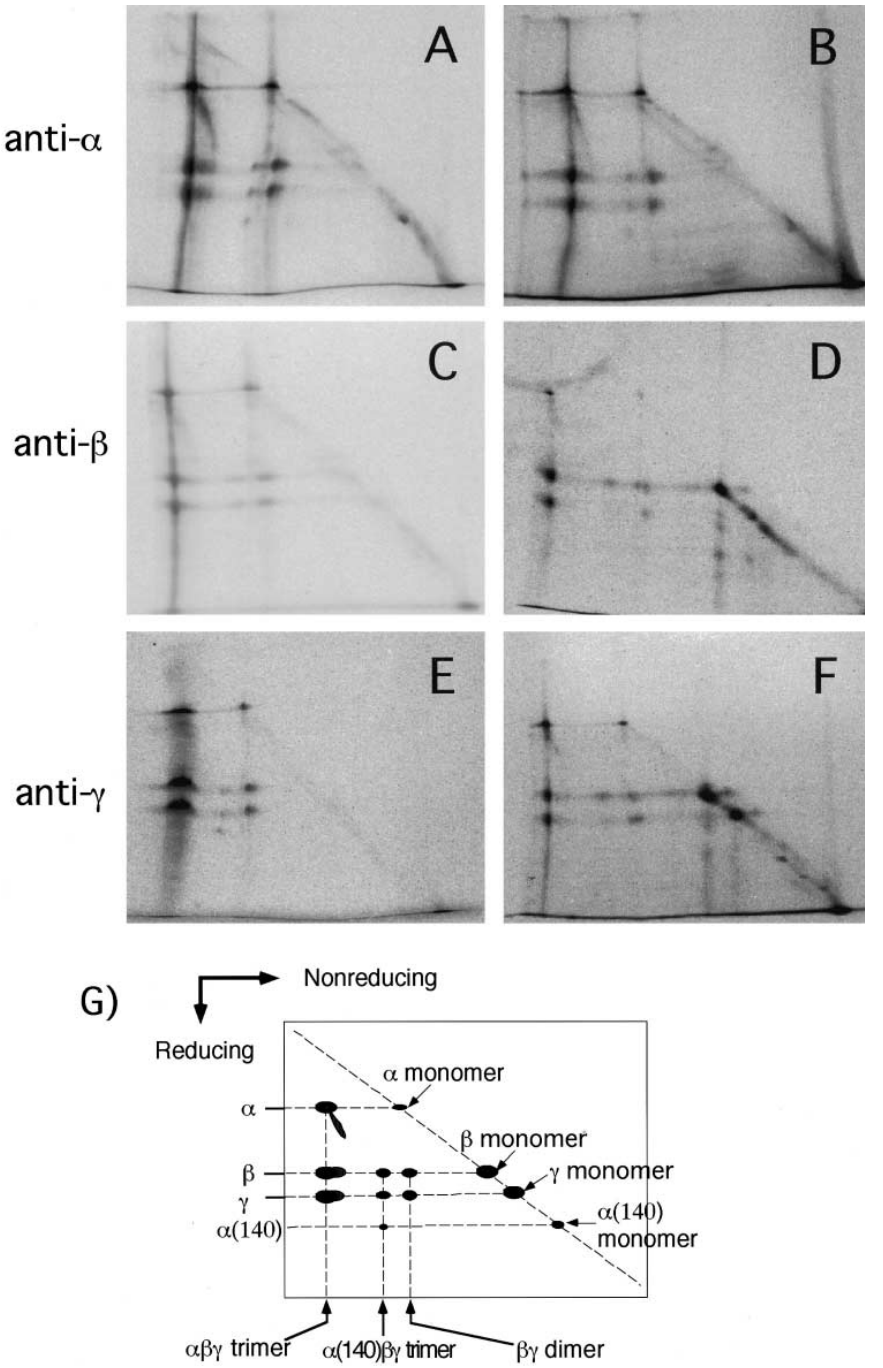


Fig. 2. Two-dimensional SDS gel electrophoresis of radiolabeled medium and cell lysate from Kc 167 cell culture. Kc 167 cells were radiolabeled with [^{35}S]methionine for 22 h and the medium (panels A, C and E) or the cell lysate (panels B, D and F) were immunoprecipitated with affinity purified anti-α antibody (A and B), anti-β antiserum (C and D), or anti-γ antiserum (E and F). Immunoprecipitates were separated by two-dimensional electrophoresis, in which non-reducing electrophoresis in the first dimension (left to right) was followed by reducing electrophoresis in the second dimension (top to bottom). Panel G is the schematic representation of the gels showing migrated positions of laminin chains.

the medium immunoprecipitated either with anti-β or -γ shows that β and γ are not secreted before disulfide-bonding.

Immunoprecipitation of radiolabeled cell lysates either with anti-α, -β or -γ antibody (Fig. 2B, D, F) confirmed that *Drosophila* laminin chains are assembled in the same order as mammalian laminin chains. Since βγ dimer is the only disulfide-bonded dimer detected, we can conclude that β and γ first form disulfide-bonded βγ dimer then α associates with this dimer to form disulfide-bonded αβγ trimer. Advantage of employing the chain-specific antibodies added following novel information. First, anti-β (anti-γ) antibody immunoprecipitates monomeric γ (β) and monomeric α in addition to monomeric β (γ), disulfide-bonded βγ dimer, disulfide-bonded αβγ trimer and a series of disulfide-bonded trimers containing β, γ and processed fragments of α (Fig. 2D or F). This result shows that β (γ) forms a stable complex with γ (β) before it is disulfide-bonded to γ (β). Immunoprecipitation of monomeric α with anti-β (γ) antibody might suggest that β (γ)

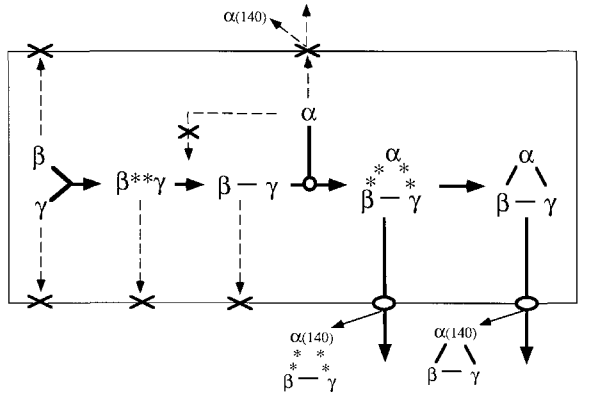


Fig. 3. The order of *Drosophila* laminin assembly in Kc 167 cells. The symbols of ** and — indicate the chain association without and with interchain disulfide-bonding.

also forms stable complex with α. Second, since anti-α antibody immunoprecipitates neither monomeric β nor γ (Fig. 2B), however, it is likely that monomeric α is immunoprecipitated with anti-β (γ) antibody due to its association with disulfide-bonded βγ dimer. At the same time, this result implies that α does not have affinity to the stable βγ dimer without disulfide-bonding. Third, anti-α antibody precipitates disulfide-bonded βγ dimer in addition to monomeric α, disulfide-bonded αβγ trimer and a series of disulfide-bonded trimers containing β, γ and processed fragments of α (Fig. 2B). These results altogether show that α associates with neither monomeric β, monomeric γ nor βγ dimer without disulfide-bonding but associates only with disulfide-bonded βγ dimer.

Experiments employing the chain-specific antibodies had minor problems concerning the stoichiometric immunoprecipitation. Probably due to unequal affinity of the antibodies to their antigens involved in various complexes, the precipitation was not always stoichiometric as typically observed in the low recovery of α(140)β1γ1 trimer with anti-β antibody (Fig. 2C and D).

Despite such minor problems, the chain-specific antibodies thus revealed the order of laminin chain assembly summarized in Fig. 3. β and γ first recognize each other and form a stable βγ dimer. β and γ are then disulfide-bonded probably through the paired cysteines at C-termini of *Drosophila* β and γ, respectively. α associates with only this disulfide-bonded βγ dimer. αβγ trimer is transported to the secretory pathway with α either disulfide-bonded or not -bonded to disulfide-bonded βγ dimer. Neither monomeric β, γ nor βγ dimer is suggested to be secreted from the cells. There remains the possibility that a small part of α is secreted probably after forming homotrimer.

3.3. *Processing of laminin α in Kc 167 cell*

Immunoprecipitates from both radiolabeled medium and cell lysate showed processed fragments of α with 140-kDa fragment as the major product. We suggest these fragments to be the processed products of α because their monomeric forms were immunoprecipitated with anti-α antibody (Fig. 2A and B) and they were assembled to disulfide-bonded βγ dimer to form a series of disulfide-bonded trimers which were immnoprecipitated either with anti-β or -γ antibody (Fig. 2C, D, E and F). This series of disulfide-bonded trimers appears on the two dimensional gel electrophoresis as three trailed spots extending from the α, β and γ spots forming the line of αβγ trimer and as three spots forming the line of α(140)βγ trimer (see the schematic representation in Fig. 2G). Trailing of α spot to the right/bottom direction of the gel implies that α chains were clipped in various domains where the cysteines for intrachain disulfide-bonding are depleted. The globular domains in the short arm and at the C-terminal are plausible sites for the processing but the long arm can also be processed as far as its ability of assembly is preserved. The 140-kDa protein seems to be the smallest and the most stable fragment among such processed products of α. These fragments are detected both in the cell lysate and the medium, suggesting that the processing occurs in the cells or at cell surface. The RIPA buffer contained PMSF, NEM and EDTA but there remained the possibility that the processing occurred during the immunoprecipitation of laminin chains.

4. Discussion

Advantage of employing chain-specific antibodies added novel information about the intracellular laminin assembly. β and γ associate with each other before they are disulfide-bonded. In contrast, α associates neither monomeric β, monomeric γ nor βγ dimer without disulfide-bonding but only with disulfide bonded βγ dimer. In experiments on de- and renaturating E8 fragments of mouse laminin 1, Engel and coworkers showed that reduced and alkylated E8β1 and E8γ1 fragments were renatured from 8 M urea to form E8β1E8γ1 dimers, and E8α1 fragment and E8β1E8γ1 dimer are able to form rod-like molecule indistinguishable from native E8 fragment [26,27]. Formation of E8β1E8γ1 dimers is consistent with the in vivo results but association of E8α1 fragment with E8β1E8γ1 is not consistent because the disulfide-bonding between E8β1 and E8γ1 was blocked by reduction and alkylation. By preparing recombinant peptides comprising of mouse α1, β1, γ1 partial sequences, Yamada and coworkers also constructed in vitro system showing the trimer formation without interchain disulfide-bonding [28,29]. These results suggest that the in vitro experiments did not precisely reflect the intracellular laminin assembly. Our in vivo experiments showed for the first time that the interchain disulfide-bonding between β and γ is crucial for the assembly of the α with the βγ dimer.

The difference of *Drosophila* laminin assembly from that of mammalian laminins [13–15] is that minor part of α can be secreted as the monomer without disulfide-bonded to βγ dimer. However, co-precipitation of disulfide-bonded βγ dimer with anti-α antibody strongly suggested that monomeric α and disulfide-bonded βγ dimer are not secreted independently. This observation is crucial to understand the phenotype of loss-of-function mutants of the *lamA* which revealed diverse functions of laminin during *Drosophila* development [30]. Since the development of the mutant proceeded beyond the first stage of laminin expression [17], there remains the possibility that functional laminin can be assembled in the absence of α. However, our observations in Kc 167 cells did not support this possibility. It is more likely that *Drosophila* has at least one more gene encoding α homologue which can substitute the function of α in the intracellular laminin assembly.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (07308073). We thank Ms. Mojgan Azimi for her technical assistance.

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