α -, β - or γ -chain-specific RNA interference of laminin assembly in *Drosophila* Kc167 cells

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Drosophila laminin $\alpha\beta\gamma$ trimer assembly in Kc167 cells was perturbed by chain-specific RNA interference (RNAi). The intracellular pool of α and γ chains remained unchanged under β -chain RNAi by lipofection of double-stranded RNA encoding a β -chain partial sequence. This was also the case for the intracellular pool of α and β chains under γ -chain-specific RNAi. Nonetheless, the intracellular pool of β and γ chains increased markedly under α -chain-specific RNAi. Non-reducing SDS/PAGE revealed that some of the increased β and γ chains migrated as disulphide-linked $\beta\gamma$ dimers but that the rest migrated

as monomers. Since the monomeric β and γ bands detected under α -chain RNAi were denser than the β band under γ -chain RNAi and the γ band under β -chain RNAi, respectively, β and γ also appeared to accumulate by forming $\beta\gamma$ dimers without the disulphide linkage. We suggest that interconversion of these $\beta\gamma$ dimers is crucial for the replaceable and selective assembly of the α chain for $\alpha\beta\gamma$ trimer formation.

Key words: basement membrane, disulphide linkage, double-stranded RNA, endoplasmic reticulum, molecular chaperone.

INTRODUCTION

Laminins [1] are a major component of basement membranes, and are composed of α , β and γ chains assembled and disulphide-linked into a cross-shaped structure with three short arms and one long arm [2–5]. In the long-arm region, each chain has many repeats of a heptad motif, which form a hydrophobic surface along the α -helix with two charged edges on each side [6–8]. Interchain hydrophobic interactions on this surface drive chain assembly, and ionic interactions at the edges determine chain selectivity. In mammals, five α chains, four β chains and three γ chains have been identified [9]. Replaceable assembly of these α chains, β chains and γ chains allows many possible $\alpha\beta\gamma$ heterotrimers to be created, but the process is at the same time selective. Our analyses of the intracellular assembly of laminin trimers in mammalian cell lines [10–12] showed that β and γ chains first form $\beta\gamma$ dimers and that then the α chain assembles with the $\beta\gamma$ dimer to form a $\alpha\beta\gamma$ trimer. Thus the $\beta\gamma$ dimer is the key intermediate in the replaceable and selective trimer assembly. For example, $\beta1\gamma1$, $\beta2\gamma1$ and $\beta3\gamma2$ are the only existing $\beta\gamma$ dimers composed of the $\beta1$, $\beta2$ or $\beta3$ chains and the $\gamma1$ or $\gamma2$ chains. The $\beta1\gamma1$ and $\beta2\gamma1$ dimers can assemble with any α chain ($\alpha1$ – $\alpha5$) to form laminin-1 ($\alpha1\beta1\gamma1$) and laminin-3 ($\alpha1\beta2\gamma1$), laminin-2 ($\alpha2\beta1\gamma1$) and laminin-4 ($\alpha2\beta2\gamma1$), laminin-6 ($\alpha3\beta1\gamma1$) and laminin-7 ($\alpha3\beta2\gamma1$), laminin-8 ($\alpha4\beta1\gamma1$) and laminin-9 ($\alpha4\beta2\gamma1$), and laminin-10 ($\alpha5\beta1\gamma1$) and laminin-11 ($\alpha5\beta2\gamma1$). On the other hand, the $\beta3\gamma2$ dimer can assemble only with the $\alpha3$ chain to form laminin-5 ($\alpha3\beta3\gamma2$). This implies that the intracellular process of $\beta\gamma$ dimer formation and its stability are critical in understanding laminin chain assembly.

By developing chain-specific antibodies and two-dimensional electrophoresis in which non-reducing and reducing SDS/PAGE are combined, we analysed laminin chain assembly in the *Drosophila* haemocyte-derived Kc167 cell line [13]. We found

that the β and γ chains form stable dimers before they are disulphide linked to each other. In contrast, the α chain (the *Drosophila* orthologue of the mammalian $\alpha5$ chain) [14] did not associate with the monomeric β chain, the monomeric γ chain or the non-disulphide-linked $\beta\gamma$ dimer, only with the disulphide-linked $\beta\gamma$ dimer, for the formation of $\alpha\beta\gamma$ trimers. Although only one β chain, one γ chain and two α chains have been found so far in *Drosophila* [15,16], further investigation into two $\beta\gamma$ dimers in Kc167 cells may provide us with a prototype mechanism for replaceable and selective assembly of the mammalian laminins.

Taking advantage of the RNA interference (RNAi) technique that we have developed recently for this cell line [17], we perturbed *Drosophila* laminin $\alpha\beta\gamma$ trimer assembly. We show here that the $\beta\gamma$ dimer accumulates as two stable forms, without and with the disulphide bond, under RNAi of α -chain synthesis. We propose that the disulphide linkage between the β and γ chains is coupled to a shift in configuration of the β and γ chains in the $\beta\gamma$ dimer from ‘dimeric’ to ‘trimeric’, and that this shift has a critical role in the replaceable and selective assembly of the α chains.

MATERIALS AND METHODS

Cell culture and lipofection of double-stranded (ds)RNAs

The *Drosophila* haemocyte-derived cell line Kc167 was maintained in HyQ CCM3 serum-free medium (Hyclone) at 25 °C. Cells (2×10^6 /well) were plated into six-well Multidish plates (Nunc) and transfected with 6 μ g of laminin α -, β - or γ -chain dsRNA using Effectene Transfection Reagent (Qiagen). Cells were also transfected in the same way with 6 μ g of odds-paired (opa) dsRNA as a negative control. opa is responsible for the determination of segmentation in *Drosophila* embryos and is not expressed in Kc167 cells, making it suitable as

Abbreviations used: ds, double stranded; ER, endoplasmic reticulum; RNAi, RNA interference; opa, odds-paired.

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Figure 1 Chain-specific RNAi of laminin α -, β - or γ -chain mRNAs in Kc167 cells

Six days after lipofection of Kc167 cells with no RNA (C), opa dsRNA or laminin α -, β - or γ -chain dsRNA, total RNA (10 μ g) was extracted, separated by 1% agarose/formaldehyde gel, blotted on to nylon membranes and hybridized with radiolabelled α -, β -, γ -chain or *Rp49* probes.

a negative control. One-fifth of each culture was removed every other day to obtain cells for further analyses.

dsRNA preparation

The template for the preparation of dsRNAs was reverse-transcription products of total RNA from Kc167 cells using random primers. They were amplified by paired primers designed to sandwich corresponding cDNA sequence between double T7 promoters (5'-TAATACGACTCACTATAGGGAGACCAC-3'). Thus opa (nt 103–1124), laminin α (nt 48–1579), laminin β (nt 424–1738) and laminin γ (nt 228–1349) cDNA fragments were amplified by PCR using the following paired primers: opa, 5'-TAATACGACTCACTATAGGGAGACCACGTAATAAGTATCAAGTCGC-3' (forward) and 5'-TAATACGACTCACTATAGGGAGACCTTGTACTTGGCCTTGAAAGGGCGTC-3' (reverse); laminin α , 5'-TAATACGACTCACTATAGGGAGACCACATGGGGACGGAGTGGCCTCCATTGGGGCTC-3' (forward) and 5'-TAATACGACTCACTATAGGGAGACCACGTTGTCACGTTGCAGTCGTTGGTAATC-3' (reverse); laminin β , 5'-TAATACGACTCACTATAGGGAGACCACATGTTGGAGCTGCGGCTTATCG-3' (forward) and 5'-TAATACGACTCACTATAGGGAGACCACGTTTCATTTAGGGAGTCACAGATG-3' (reverse); and laminin γ , 5'-TAATACGACTCACTATAGGGAGACCACATGAAGCGCAGCCGGTGGAGCC-3' (forward) and 5'-TAATACGACTCACTATAGGGAGACCACGATTTCGCGTCAAGAAGCAC-3' (reverse). Amplified cDNAs were transcribed by T7 RNA polymerase (Promega). The RNA products were treated with RNase-free DNase and purified by phenol/chloroform extraction and ethanol precipitation. Although the RNA products could be self-annealed, the annealing was completed by dissolving precipitated RNAs in annealing

buffer (2 mM Tris/HCl, pH 7.5/2 mM EDTA), boiling for 2 min and allowing to stand at room temperature for 12 h.

Northern blotting

Total RNA was extracted from the transfected cells using Trizol reagent (Gibco-BRL). RNAs corresponding to 10 μ g were separated in 1.0% agarose/formaldehyde gels, blotted on to Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech) and hybridized to ³²P-labelled laminin α (nt 48–1579), β (nt 424–1738) or γ (nt 228–1349) or *Rp49* cDNA probes at 42 °C in 5 × SSPE (0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) containing 0.5% SDS, 5 × Denhardt's solution, 50% formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC/0.1% SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at room temperature and twice with 0.1 × SSC/0.1% SDS at 55 °C for 30 min. Analysis was carried out using a Fuji Film BAS 2000 Image Analyser.

Immunoblotting

After lipofection with dsRNA (2, 4 or 6 days), 2 × 10⁶ cells were lysed in reducing or non-reducing SDS sample buffer (3% SDS, 67 mM Tris/HCl, pH 6.8, 5% glycerol, 0.003% Bromophenol Blue and with or without 2% 2-mercaptoethanol, respectively). Proteins in the culture media conditioned with the same number of cells for 4 days were dissolved in the same SDS sample buffers after precipitation in 10% trichloroacetic acid. Proteins in the cell lysates and conditioned media were separated by SDS electrophoresis using 4% polyacrylamide gel, and proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with PBS containing 5%

skimmed milk for 90 min, incubated with antiserum against laminin β or γ chains (1:1000 dilutions) for 16 h at 4 °C, washed extensively with PBS containing 0.1 % Tween-20 and reacted with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 dilution) for 1 h at room temperature. For immunoblotting of the α chain, an affinity-purified antibody [13] was used. The ECL Western-blotting system (Amersham Pharmacia Biotech) was used for detection.

RESULTS

Chain-specific RNAi by lipofection of Kc167 cells with α -, β - or γ -chain dsRNA

Northern blotting of total RNA from the cells 6 days after lipofection with α -, β - or γ -chain dsRNA showed specific reduction of the corresponding mRNAs to less than one-tenth (Figure 1). This RNAi could be observed within 2 days of the lipofection and continued at least for 8 days (results not shown). The two bands detected for the α chain, compared with one band each for β and γ , suggest partial cleavage of the intact mRNA, and both diminished under RNAi. The specificity of RNAi was double checked by confirming that lipofection of opa dsRNA did not affect α -, β - or γ -chain mRNAs and that Rp49 mRNA

Figure 2 Immunoblot of laminin chains in Kc167 cells after chain-specific RNAi

After lipofection (4 days) of Kc167 cells with no RNA (C), opa or laminin α -, β - or γ -chain dsRNA, lysates from 2×10^6 cells were separated by SDS/PAGE under reducing conditions and immunoblotted with affinity-purified antibody against α chain (Anti- α), or antiserum against β chain (Anti- β) or γ chain (Anti- γ). Despite affinity purification, the anti- α -chain antibody recognized many bands with faster mobility than the α chain. Co-migration of *Drosophila* laminin α chain purified from the conditioned medium of Kc167 cells confirmed that the band indicated by arrows (in the top panel) is the α chain. Probably due to shared epitope structures, anti- β - and anti- γ -chain antisera also recognized the γ and β chains, respectively, as minor bands in addition to the major bands that are indicated by arrows (middle and bottom panels).

Figure 3 Immunoblot of laminin chains secreted from Kc167 cells after chain-specific RNAi

After lipofection (4 days) of Kc167 cells with no RNA (C), opa or laminin α -, β - or γ -chain dsRNA, the media conditioned from 2×10^6 cells were separated by SDS/PAGE under reducing conditions. Other details were same as in Figure 2.

was not affected by lipofection with α -, β - or γ -chain dsRNAs. The fact that, under extensive degradation of mRNA for any one of the chains (α -, β - or γ -), mRNA levels for the other two chains are unaffected suggested that the α -, β - and γ -chain genes are transcribed without any regulatory mechanism for balancing the chain pool. Northern blotting incidentally revealed that large amounts of introduced dsRNA and its degradation products even remained in the cells 6 days after lipofection.

Immunoblotting of cell lysates 4 days after lipofection with chain-specific antibody following reducing SDS/PAGE confirmed that the intracellular pools of α chain, β chain and γ chain (Figure 2, top, middle and bottom panels, respectively) were depleted in response to the degradation of corresponding mRNA.

β and γ chains accumulate on depletion of the α chain

Figure 2 also depicts the consequences of depletion of one laminin chain on the other chains. Depletion of the β chain marginally reduced the α -chain (Figure 2, top panel) and γ -chain (Figure 2, bottom panel) pools, and depletion of the γ chain did not affect the α -chain (Figure 2, top panel) or β -chain (Figure 2, middle panel) pools. These results suggested that the steady-state level of β and γ monomers was maintained, probably by degradation balanced with synthesis, under the depletion of the assembly partners for $\beta\gamma$ dimer formation. The same was true for the steady-state levels of α monomer under the depletion of $\beta\gamma$ dimer, which is needed for $\alpha\beta\gamma$ trimer formation. In contrast, a marked increase in β (Figure 2, middle panel) and γ (Figure 2, bottom panel) chains was observed on depletion of the α chain.

Figure 4 Immunoblot of laminin chains in Kc167 cells after chain-specific RNAi separated by non-reducing SDS/PAGE

The same cell lysates as in Figure 2 were separated by SDS/PAGE under non-reducing conditions and immunoreacted with antisera against β chain (Anti- β) or γ chain (Anti- γ). Other details were same as in Figure 2. Because of its extremely large size (≈ 800 kDa), the $\alpha\beta\gamma$ trimer could not be transblotted.

This suggests that the β and γ chains can stabilize themselves and that their accumulation is enhanced by depletion of the α -chain pool. Essentially the same results were obtained from the cells 2 and 6 days after lipofection, except that the γ chain tended to be stabilized by longer durations of β -chain depletion (results not shown).

In order to know the consequence of depletion of one laminin chain on the secretion of the others, the conditioned media were also analysed (Figure 3). A clear contrast with the observations in the cell lysates was that RNAi of the synthesis of any chain reduced secretion of all chains in a parallel manner. This shows that laminin chains can leave the endoplasmic reticulum (ER) lumen for the secretion pathway only after $\alpha\beta\gamma$ trimer formation, as is the case in mammalian cells [10–12]. The parallel reduction of all chains under RNAi of synthesis of any chain suggests that neither monomeric nor dimeric laminin chains can be secreted. Secretion of small amounts of laminin chains under RNAi was probably due to incomplete interference. Small intracellular pools of the chains remained in spite of RNAi, allowing secretion of the other chains after the formation of limited amounts of the $\alpha\beta\gamma$ trimer.

Two stable forms of the $\beta\gamma$ dimer, with and without the disulphide linkage

To analyse the disulphide linkage between β and γ chains, the same cell lysates were separated by non-reducing SDS/PAGE and immunoblotted with anti- β -chain (Figure 4, left-hand panel) or anti- γ -chain (Figure 4, right-hand panel) antibodies. Whereas the disulphide-linked $\beta\gamma$ dimer diminished in the cells transfected with β - or γ -chain dsRNA, it increased in the cells transfected with α -chain dsRNA. In addition, the β and γ bands migrating as monomers were denser in the cells transfected with α -chain dsRNA compared with cells transfected with β - or γ -chain dsRNA. Since pull-down experiments of the γ (β) chain with an anti- β (γ) chain antibody substantiated the $\beta\gamma$ dimer without the interchain disulphide bond [13], part of the monomeric β (γ) chain was likely to be stabilized by forming this $\beta\gamma$ dimer. Thus, the results in Figure 4 show that β and γ chains accumulated

in the α -chain-depleted cells by forming $\beta\gamma$ dimers with and without the disulphide linkage. Rough estimations suggested that more β and γ chains were involved in the latter type of $\beta\gamma$ dimer than in the former.

DISCUSSION

Chain-specific depletion by RNAi revealed the kinetics of laminin chain assembly in Kc167 cells. Depletion of β or γ chains left the pool sizes of partner chains unchanged (Figure 2). This indicates that the low solubility of monomeric chains due to their hydrophobic surfaces for chain assembly limits the rate of the laminin chain assembly under normal conditions. In contrast, depletion of α chains caused accumulation of β and γ chains, consistent with the mechanism of $\beta\gamma$ dimer formation occurring before $\alpha\beta\gamma$ trimer assembly. Limited pool sizes of monomeric β and γ chains may be expanded by the neutralization of their hydrophobic surfaces that occurs on formation of $\beta\gamma$ dimers. $\beta\gamma$ dimers thus accumulated are ready to associate with α chains to form $\alpha\beta\gamma$ trimers. It is intriguing that the most critical step in trimer assembly is overcome by such a mechanism of intermediate stabilization. This implies that laminin chains are their own molecular chaperones and explains why we could not specify a molecular chaperone for laminin chains [18].

Expansion of the $\beta\gamma$ -dimer pool under α -chain depletion revealed two forms of $\beta\gamma$ dimer; one without and one with the interchain disulphide bond. A simultaneous increase of both forms by α -chain depletion suggested that the equilibrium between them is probably mediated by protein disulphide-isomerase, which is abundant in the ER lumen. Since immunoprecipitation with an anti- α -chain antibody showed that α chains associate only with disulphide-linked $\beta\gamma$ dimers [13], the role of the more abundant non-disulphide-linked $\beta\gamma$ dimer needs to be explained. In the model depicted in Figure 5, we would like to propose that the 'dimeric' and 'trimeric' configurations of β and γ chains in $\beta\gamma$ dimers [6] are coupled to the interchain disulphide bond. In order to accept an α chain as the third chain, the preferable dimeric configuration of β and γ chains in the $\beta\gamma$ dimer needs to shift to the trimeric configuration by rotation of

Figure 5 Interconversion of 'dimeric' and 'trimeric' configurations of β and γ chains in two $\beta\gamma$ dimers is coupled to the interchain disulphide linkage

Coiled-coil association of laminin chains by the heptad repeats of $(abcdefg)_n$ in the long-arm regions is schematically depicted. Hydrophobic amino acids often appear at the a and d positions and drive the chain association. Charged amino acids often appear at the e and g positions and determine the chain selectivity. The shadowed area indicates interchain hydrophobic interaction and dotted lines indicate interchain ionic interactions. In ER, solubility of monomeric α , β and γ chains is limited and excess monomers are continuously degraded. Monomeric β and γ chains associate with each other to form the $\beta\gamma$ dimer in the dimeric configuration. The configuration of the $\beta\gamma$ dimer then shifts to trimeric, in association with the interchain disulphide linkage. The monomeric α chain associates only with the $\beta\gamma$ dimer in the trimeric configuration to form $\alpha\beta\gamma$ trimer, which leaves the ER for the secretion pathway. If the $\beta\gamma$ dimer in its trimeric configuration remains in the ER due to mismatching, it shifts back to the dimeric configuration for another round of trimerization.

the β and γ chains relative to each other, and this unfavourable configuration is stabilized by the interchain disulphide linkage. Once the trimers are formed correctly, they may leave the ER lumen for the secretion pathway. In the case of mismatching, however, mal-folded trimers are arrested in the ER lumen due to the yet-to-be-neutralized hydrophobic surface. To avoid suicidal accumulation of mal-folded laminin chains, protein disulphide-isomerase, which is abundant in the ER lumen, may hydrolyse the disulphide linkage for the recruitment of $\beta\gamma$ dimers into the dimeric configuration for another round of trimerization. Such a shift of β and γ configuration may be needed for every heptad motif repeated along the whole long-arm region. By preparing a variety of recombinant laminin chains, Utani et al. [19,20] showed that assembly at the C-terminus of the long arm is crucial for triggering the process and that assembly of the whole long arm proceeds from the C- to the N-terminus, like sliding a zip. It is intriguing that the disulphide linkage between β and γ chains is located very close to this triggering site.

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