



RESEARCH & REVIEW

## Disulfide Bond Formation of Heterodimer and Heterotrimer of Human Laminin-332 Coiled-coil Domains

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ARTICLE INFO

Editor:

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Keywords:

laminin-332  
laminin assembly  
coiled-coil domain  
disulfide bond  
cell-free translation  
chaperone

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ABSTRACT

Laminin (LM)  $\alpha$ ,  $\beta$ , and  $\gamma$  chains were connected by disulfide bonds at the C- and N-termini of the LM coiled-coil (LCC) domain to form heterodimers and heterotrimers. At the C-terminus of LCC domain, one disulfide bond is formed to connect  $\beta$  and  $\gamma$  chains while it was unclear how disulfide bond pattern is formed to connect  $\alpha$ ,  $\beta$ , and  $\gamma$  chains at the N-terminus of LCC domain. Using an insect cell-free translation system, we succeeded to produce heterotrimers of LCC domain of human LM-332. To analyze disulfide bond formation at the N-terminus of LCC domain, we mutated cysteines of LCC domains into alanines by site-directed mutagenesis and co-expressed these mutants in an insect cell-free translation system. Mutation of a single cysteine at the N-terminus of LCC domain of one chain caused the failure of disulfide bond formation of heterotrimers. However, mutation of cysteines at the N-terminus of LCC domain of two different chains recovered the disulfide bond formation of heterotrimers with different efficiencies. These results suggest that the disulfide bond patterns at the N-terminus of human LM-332 LCC domains are not specific.

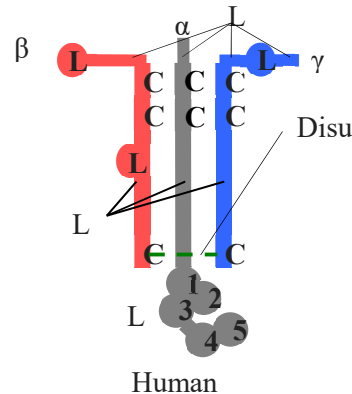
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## 1. Introduction

Laminins (LM) are large multidomain glycoproteins of the extracellular matrix (ECM). LMs and type IV collagen, nidogen, perlecan, agrin are major components of basement membranes (BM) that act as supportive architecture for the cells to proliferate, differentiate, and migrate. Each LM molecule is a 500 to 900 kDa heterotrimeric glycoprotein, in which  $\alpha$ ,  $\beta$ , and  $\gamma$  chains are assembled and disulfide bonded in a cross shaped structure with three short arms and one rodlike long arm (1-4). Since the first purification of LM-111 from mouse Engelbreth-Holm-Swarm (EHS) sarcoma (5), five  $\alpha$ , three  $\beta$ , and three  $\gamma$  chains have been recognized to combine into 16 different heterotrimeric LMs (6).

The LM coiled-coil (LCC) domain is the site of trimer assembly. It has many repeats of the heptad motif where hydrophobic residues are located in the first and fourth positions and charged residues in the fifth and seventh positions (7-9). They form a hydrophobic surface along  $\alpha$ -helix with ionic edges at both sides. Interchain hydrophobic interactions at this surface drive the chain assembly and ionic interactions at the edges determine the chain selectivity (10). Many studies of LM trimer assembly confirmed that  $\beta$  and  $\gamma$  chains first form  $\beta$ - $\gamma$  heterodimer with disulfide bonds. Then,  $\alpha$  chain assembles with  $\beta$ - $\gamma$  heterodimer to form  $\alpha$ - $\beta$ - $\gamma$  heterotrimer with disulfide bonds (11-14). Two cysteine residues (-C-X-X-C- motif) at the N-terminus and one cysteine residue at the C-terminus of LCC domains involve in disulfide bond formation of  $\beta$ - $\gamma$  heterodimers and  $\alpha$ - $\beta$ - $\gamma$  heterotrimers (2, 12, 15-17). Up to now, we do not know exactly which cysteine forms disulfide bond with which cysteine at the N-terminus of LCC domains.

In our previous study, we succeeded to produce heterotrimers of LCC domain of human LM-332 ( $\alpha 3\beta 3\gamma 2$ ) in an insect cell-free translation system (17). In this study, we mutated cysteine residues at the N- and C-termini of the LCC domains of human LM-332 into alanine residues by site-directed mutagenesis and co-expressed these mutants to analyze the role of these cysteine residues for LM heterotrimer formation with disulfide bonds *in vitro* (Fig. 1). The results showed that disulfide bonds at the N-terminus of heterotrimers of LCC domains could not form when we mutated only a single cysteine residue at the N-terminus of LCC domain of one chain into alanine residue. But the disulfide bonds could form with different efficiencies when we mutated cysteine residues at the N-terminus of LCC domain of two different chains into alanine residues. These results suggest that there is no fixed disulfide bond pattern among six cysteine residues at the N-terminus of LCC domains of human LM-332 heterotrimers.



**Fig. 1.** Model of human LM-332 heterotrimer. Human LM-332 consists of  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  chains;  $\alpha 3$  chain has an LM epidermal growth factor-like (LEc1-4) domain, an LM coiled-coil (LCC) domain, and an LM globular (LGI-5) domain;  $\beta 3$  chain has an LM N-terminal (LN) domain, an LEa1-3 domain, an LEB1-3 domain, an LCC domain, and an LM  $\beta$  knob (L $\beta$ ) domain;  $\gamma 2$  chain has an LEa1-3 domain, an LM 4 (L4) domain, an LEB1-3 domain, and an LCC domain. LCC domains of  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  chains assemble to form heterotrimers by disulfide bonds at the N- and C-termini. The drawing is to show that the disulfide bonds exist but does not imply their specificity.

## 2. Materials and Methods

### 2.1 Plasmid construction

We had cloned the LCC domains of human LM  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  chains into pTD1 vectors as previously described (17-20). We used these vectors as templates for site-directed mutagenesis using GeneTailor site-directed mutagenesis kit (Invitrogen, Carlsbad, CA). We designed primers as shown in Table I. Mutated plasmids were checked by sequencing with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). The mutated LCC domains were named by adding their mutated cysteine residues  $C^1$ ,  $C^2$ , and  $C^3$  into their names. Thus, single mutations of LCC domains were named as  $\alpha 3C^1$ ,  $\alpha 3C^2$ ,  $\beta 3C^1$ ,  $\beta 3C^2$ ,  $\beta 3C^3$ ,  $\gamma 2C^1$ ,  $\gamma 2C^2$ , and  $\gamma 2C^3$  while double mutations were named as  $\beta 3C^1C^2$ ,  $\beta 3C^1C^3$ ,  $\beta 3C^2C^3$ , and  $\gamma 2C^2C^3$ . One special mutant of the  $\alpha 3$  LCC domain was made by substituting valine residue Val217 of  $\alpha 3C^2$  mutant with a cysteine. This special mutant was named as  $\alpha 3C^*$  (Fig. 2).

**Table I.** List of primers used for site-directed mutagenesis

Mutant names	Primer sequences	Template
$\alpha 3C^1$	5'-ATGACGATGACAAGGATGATGCCGACAGCTGTG-3';5'-ATCATCCTTGTCATCGTCATCCTTGTAGTC-3'	$\alpha 3$ LCC
$\alpha 3C^2$	5'-ACAAGGATGATTGCGACAGCGCTGTGATGACCC-3';5'-GCTGTGCAATCATCCTTGTCATCGTCATC-3'	$\alpha 3$ LCC
$\alpha 3C^*$	5'-ACAAGGATGATTGCGACAGCGCTTGCATGACCCTCC-3';5'-GCTGTGCAATCATCCTTGTCATCGTCATC-3'	$\alpha 3C^2$
$\beta 3C^1$	5'-AATATAAAGATATGGTGGCCGCCACCCTTGCT-3';5'-GGCCACCATATCTTTATATTTTATTTTCT-3'	$\beta 3$ LCC
$\beta 3C^2$	5'-ATATGGTGGCCTGCCACCCTGCCTCCAGACCT-3';5'-AGGGTGGCAGGCCACCATATCTTATATTT-3'	$\beta 3$ LCC
$\beta 3C^3$	5'-GCGTGCTCTACTATGCCACCGCCAAGTAGTCT-3';5'-GGTGGCATAGTAGAGCACGCGCCCATGAT-3'	$\beta 3$ LCC
$\beta 3C^1C^2$	5'-AATATAAAGATATGGTGGCCGCCACCCTGCC-3';5'-GGCCACCATATCTTTATATTTTATTTTCT-3'	$\beta 3C^2$
$\beta 3C^1C^3$	5'-GCGTGCTCTACTATGCCACCGCCAAGTAGTCT-3';5'-GGTGGCATAGTAGAGCACGCGCCCATGAT-3'	$\beta 3C^1$
$\beta 3C^2C^3$	5'-GCGTGCTCTACTATGCCACCGCCAAGTAGTCT-3';5'-GGTGGCATAGTAGAGCACGCGCCCATGAT-3'	$\beta 3C^2$
$\gamma 2C^1$	5'-TGGAGCATGGAGCATTACGCGCTCCAGCTTGCT-3';5'-GCTGAATGCTCCATGCTCCATATCTTATA-3'	$\gamma 2$ LCC
$\gamma 2C^2$	5'-GAGCATTAGCTGTCCAGCTGCCTATAATCAAG-3';5'-AGCTGGACAGCTGAATGCTCCATGCTCCAT-3'	$\gamma 2$ LCC
$\gamma 2C^3$	5'-GGGACAACCTGCCCCAGGCGCTACAATACCC-3';5'-GCCTGGGGCAGGTTGTCCCTAATGTTCTC-3'	$\gamma 2$ LCC
$\gamma 2C^2C^3$	5'-GGGACAACCTGCCCCAGGCGCTACAATACCC-3';5'-GCCTGGGGCAGGTTGTCCCTAATGTTCTC-3'	$\gamma 2C^2$

FLAG- <b>DDC<sup>1</sup>DSC<sup>2</sup>VM</b>	<b>EDLKGY</b>	$\alpha 3$
FLAG- <b>DDA<sup>1</sup>DSC<sup>2</sup>VM</b>	<b>EDLKGY</b>	$\alpha 3C^1$
FLAG- <b>DDC<sup>1</sup>DSA<sup>2</sup>VM</b>	<b>EDLKGY</b>	$\alpha 3C^2$
FLAG- <b>DDC<sup>1</sup>DSA<sup>2</sup>C<sup>3</sup>M</b>	<b>EDLKGY</b>	$\alpha 3C^*$
<b>VAC<sup>1</sup>HPC<sup>2</sup>FQ</b>	<b>YYATC<sup>3</sup>K</b>	$\beta 3$
<b>VAA<sup>1</sup>HPC<sup>2</sup>FQ</b>	<b>YYATC<sup>3</sup>K</b>	$\beta 3C^1$
<b>VAC<sup>1</sup>HPA<sup>2</sup>FQ</b>	<b>YYATC<sup>3</sup>K</b>	$\beta 3C^2$
<b>VAC<sup>1</sup>HPC<sup>2</sup>FQ</b>	<b>YYATA<sup>3</sup>K</b>	$\beta 3C^3$
<b>VAA<sup>1</sup>HPA<sup>2</sup>FQ</b>	<b>YYATC<sup>3</sup>K</b>	$\beta 3C^1C^2$
<b>VAA<sup>1</sup>HPC<sup>2</sup>FQ</b>	<b>YYATA<sup>3</sup>K</b>	$\beta 3C^1C^3$
<b>VAC<sup>1</sup>HPA<sup>2</sup>FQ</b>	<b>YYATA<sup>3</sup>K</b>	$\beta 3C^2C^3$
<b>EHGAFSC<sup>1</sup>PAC<sup>2</sup></b>	<b>C<sup>3</sup>YNTQALEQQ</b>	$\gamma 2$
<b>EHGAFSA<sup>1</sup>PAC<sup>2</sup></b>	<b>C<sup>3</sup>YNTQALEQQ</b>	$\gamma 2C^1$
<b>EHGAFSC<sup>1</sup>PAA<sup>2</sup></b>	<b>C<sup>3</sup>YNTQALEQQ</b>	$\gamma 2C^2$
<b>EHGAFSC<sup>1</sup>PAC<sup>2</sup></b>	<b>A<sup>3</sup>YNTQALEQQ</b>	$\gamma 2C^3$
<b>EHGAFSC<sup>1</sup>PAA<sup>2</sup></b>	<b>A<sup>3</sup>YNTQALEQQ</b>	$\gamma 2C^2C^3$

**Fig. 2.** Summary of LCC domains of human LM-332 and their mutants used in this study. A FLAG tag was inserted at the N-terminus of LCC domain of  $\alpha 3$  chain. A part of amino acid sequences from the N- and C-termini of LCC domains and their mutants were showed in boxes. Two cysteine residues at the N-terminus ( $-C^1-X-X-C^2-$ ) and one cysteine residue at the C-terminus ( $C^3$ ) of LCC domains were mutated into alanine residues (A) by site-directed mutagenesis. The mutants were named by adding their mutated cysteine residues ( $C^1$ ,  $C^2$ , and  $C^3$ ) into their names. One special mutant,  $\alpha 3C^*$ , was created from  $\alpha 3C^2$  by mutating the valine residue that is adjacent to the alanine residue into cysteine. In this study, we called  $\alpha 3C^1$ ,  $\alpha 3C^2$ ,  $\beta 3C^1$ ,  $\beta 3C^2$ ,  $\beta 3C^3$ ,  $\gamma 2C^1$ ,  $\gamma 2C^2$ , and  $\gamma 2C^3$  as single cysteine mutants, and  $\beta 3C^1C^2$ ,  $\beta 3C^1C^3$ ,  $\beta 3C^2C^3$ , and  $\gamma 2C^2C^3$  as double cysteine mutants.

## 2.2 Protein expression

mRNAs were synthesized from 1  $\mu$ g of the linearized vectors using a ScriptMax Thermo T7 Transcription Kit (Toyobo, Osaka, Japan). The synthesized mRNAs were diluted to a final concentration of 2  $\mu$ g/ $\mu$ l with nuclease-free water. Ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 1 mM. Protein synthesis was carried out using a Transdirect insect cell kit (Shimadzu, Kyoto, Japan) in non-reducing conditions. To check the heterodimer and heterotrimer formation, 6  $\mu$ g of mRNAs of each  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  LCC domains and their mutants were mixed with other reagents of the kit to

reach 50  $\mu$ l of reaction volume following instruction (21-23). The reaction mixtures were incubated for 5 h at 25  $^{\circ}$ C.

## 2.3 Western blot analysis

Protein samples were separated on 8% sodium dodecyl sulphate (SDS) polyacrylamide gels under reducing or non-reducing conditions, and transferred onto Hybond<sup>TM</sup>-ECL membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% non-fat dry milk in phosphate buffer saline (PBS) buffer containing 0.1% Tween-20 (blocking buffer) for an hour at room temperature. Next, the membranes were incubated with primary antibodies against FLAG-tag (1:2,000) (monoclonal anti-FLAG M2; Sigma, St. Louis, MO), against LM  $\beta 3$  (1:2,000) (polyclonal antibody H-300; Santa Cruz Biotechnology, Santa Cruz, CA), against LM  $\gamma 2$  (1:2,000) (monoclonal antibody B-2; Santa Cruz Biotechnology) in blocking buffer for 1 hour at room temperature. Membranes were washed with PBS buffer containing 0.1% Tween-20 (washing buffer), and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000) (GE Healthcare) in blocking buffer for 1 hour at room temperature. Membranes were washed again in washing buffer and developed using ECL western blotting detection reagents (GE Healthcare), and visualized on Hyperfilm MP (GE Healthcare).

## 3. Results

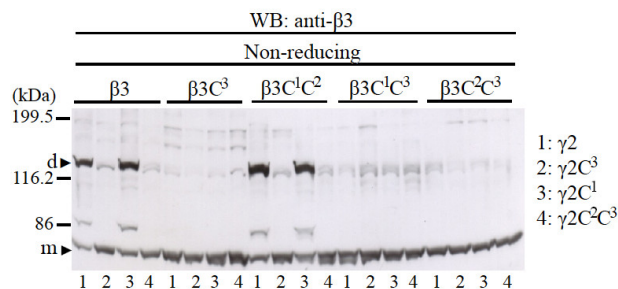
### 3.1 Mutation of cysteine residues of LCC domains of human LM-332 into alanine residues by site-directed mutagenesis

One cysteine residue at the C-terminus of  $\beta$  and  $\gamma$  LCC domains and two cysteine residues at the N-terminus of  $\alpha$ ,  $\beta$ , and  $\gamma$  LCC domains are necessary for disulfide bond formation of LM heterodimer and heterotrimer (2). These cysteine residues are Cys213 and Cys216 at the N-terminus of  $\alpha 3$  LCC; Cys581 and Cys584 at the N-terminus and Cys1177 at the C-terminus of  $\beta 3$  LCC; Cys609 and Cys612 at the N-terminus and Cys1190 at the C-terminus of  $\gamma 2$  chain. To explain our data easily, we called Cys213 and Cys216 of the  $\alpha 3$  chain, Cys581 and Cys584 of the  $\beta 3$  chain, Cys609 and Cys612 of the  $\gamma 2$  chain as  $C^1$  and  $C^2$  of the corresponding chains, respectively. We also called Cys1177 of the  $\beta 3$  chain and Cys1190 of the  $\gamma 2$  chain as  $C^3$  of the  $\beta 3$  chain and  $C^3$  of the  $\gamma 2$  chain (Fig. 1). We created single cysteine mutations of LCC domains including  $\alpha 3C^1$ ,  $\alpha 3C^2$ ,  $\alpha 3C^*$ ,  $\beta 3C^1$ ,  $\beta 3C^2$ ,  $\beta 3C^3$ ,  $\gamma 2C^1$ ,  $\gamma 2C^2$ , and  $\gamma 2C^3$ . We also created double cysteine mutations of LCC domains including  $\beta 3C^1C^2$ ,  $\beta 3C^1C^3$ ,  $\beta 3C^2C^3$ , and  $\gamma 2C^2C^3$  (Fig. 2). We co-expressed these mutated  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  LCC

domains with normal  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  LCC domains in different combinations to check disulfide bond formation of heterodimers and heterotrimers.

### 3.2 Disulfide bond formation at the N- and C-termini of heterodimer of $\beta 3$ and $\gamma 2$ LCC domains

First, we investigated the roles of two cysteine residues,  $C^1$  and  $C^2$ , at the N-terminus and one cysteine residue,  $C^3$ , at the C-terminus of  $\beta 3$  and  $\gamma 2$  LCC domains for disulfide bond formation of  $\beta 3$ - $\gamma 2$  heterodimer. Many experiments confirmed that LM  $\beta$  and  $\gamma$  chains form a disulfide bond with cysteine  $C^3$  at the C-terminus of LCC domains (7, 11, 15). However, cysteines  $C^1$  and  $C^2$  at the N-terminus of LCC domain of  $\beta$  and  $\gamma$  chains have not been reported to form disulfide bonds. We co-expressed  $\beta 3$  or  $\beta 3C^3$ ,  $\beta 3C^1C^2$ ,  $\beta 3C^1C^3$ , and  $\beta 3C^2C^3$  with  $\gamma 2$  or  $\gamma 2C^3$ ,  $\gamma 2C^1$ , and  $\gamma 2C^2C^3$  to examine if disulfide bonds by cysteines  $C^1$  and  $C^2$  at the N-terminus can be formed in the absence of cysteine  $C^3$  at the C-terminus LCC domains. We also examined which cysteine of  $\beta 3$  LCC binds to which cysteine of  $\gamma 2$  LCC at the N-terminus in the case  $\beta 3$  and  $\gamma 2$  LCCs can form disulfide bonds at the N-terminus. Western blotting using anti- $\beta 3$  antibody showed that when  $\beta 3$  and  $\gamma 2$  LCCs did not have cysteine  $C^3$  at the C-terminus, heterodimer could not form disulfide bond by cysteine  $C^1$  and  $C^2$  at the N-terminus (Fig. 3). Western blotting using anti- $\gamma 2$  antibody showed the same results (data not shown). Therefore, the disulfide bond of cysteines  $C^3$  at the C-terminus of LCC domain of  $\beta 3$  and  $\gamma 2$  chains is crucial for  $\beta 3$ - $\gamma 2$  heterodimer formation without  $\alpha 3$  LCC domain; in the absence of cysteine  $C^3$ , disulfide bonds of cysteines  $C^1$  and  $C^2$  at the N-terminus of LCC domains did not form.

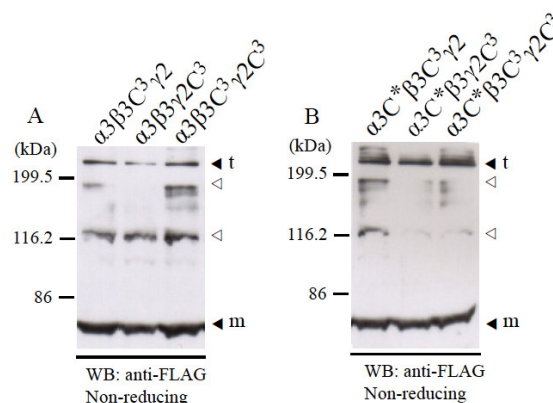


**Fig. 3.** The role of cysteine residues  $C^1$ ,  $C^2$ , and  $C^3$  of LCC domains of  $\beta 3$  and  $\gamma 2$  chains for disulfide bond formation of  $\beta 3$ - $\gamma 2$  heterodimer. Mutants of  $\beta 3$  chain ( $\beta 3$ ,  $\beta 3C^3$ ,  $\beta 3C^1C^2$ ,  $\beta 3C^1C^3$ , and  $\beta 3C^2C^3$ ) were co-expressed with mutants of  $\gamma 2$  chain ( $\gamma 2$ ,  $\gamma 2C^3$ ,  $\gamma 2C^1$ , and  $\gamma 2C^2C^3$ ). Samples were load into an 8% SDS-PAGE gel, in non-reducing condition and immunoblotted with an anti- $\beta 3$  antibody. The results showed that only when both LCC domains of  $\beta 3$  and  $\gamma 2$  chains had cysteine  $C^3$  at the C-terminus,  $\beta 3$ - $\gamma 2$  heterodimers could be formed (lanes 1

and 3 of  $\beta 3$  and  $\beta 3C^1C^2$ ). Molecular weight markers are indicated on the left. Monomer (m) and dimer (d) bands are indicated with black triangles.

### 3.3 Disulfide bond formation with cysteines $C^1$ and $C^2$ at the N-terminus of LCC domains of $\alpha 3$ - $\beta 3$ - $\gamma 2$ heterotrimer

To check for disulfide bond formation by cysteines  $C^1$  and  $C^2$  at the N-terminus of LCC domains, we co-expressed LCC domain of  $\alpha 3$  chain with  $\beta 3C^3$  and  $\gamma 2C^3$  mutants which do not have cysteine  $C^3$  at the C-terminus but intact cysteines  $C^1$  and  $C^2$  at the N-terminus. Western blotting using an anti-FLAG antibody showed that  $\beta 3C^3$  and  $\gamma 2C^3$  mutants could form heterotrimers with disulfide bonds normally (Fig. 4A). Western blotting using anti- $\beta 3$  antibody and anti- $\gamma 2$  antibody show similar results (data not shown). Therefore, without disulfide bond at C-terminus of LCC domain of  $\beta 3$  and  $\gamma 2$  chains,  $\alpha 3$ - $\beta 3$ - $\gamma 2$  heterotrimer could form disulfide bonds by cysteines  $C^1$  and  $C^2$  at the N-terminus of LCC domains.

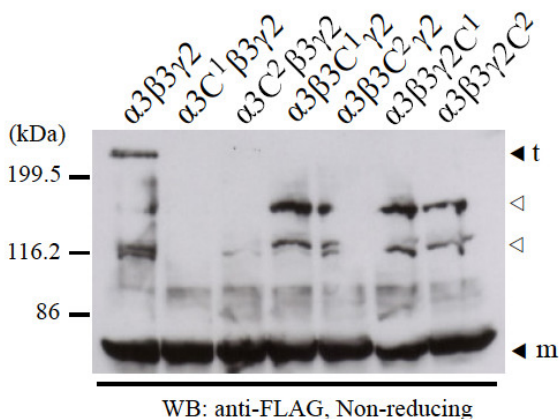


**Fig. 4.** Disulfide bond formation of  $\alpha 3$ - $\beta 3$ - $\gamma 2$  heterotrimer with cysteines  $C^1$  and  $C^2$  at the N-termini of LCC domains. LCC domains of  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  chains and their mutants ( $\alpha 3C^*$ ,  $\beta 3C^3$ , and  $\gamma 2C^3$ ) were co-expressed and checked for disulfide bond formation at the N-terminus. Expression samples were load into an 8% SDS-PAGE gel in non-reducing condition and detected by western blot with an anti-FLAG antibody. A, LCC domains of  $\alpha 3$  chain could form disulfide bonds with  $\beta 3C^3$  and  $\gamma 2C^3$  mutants at the N-terminus. B,  $\alpha 3C^*$  mutant which has the -C-X-X-X-C- motif at the N-terminus could form disulfide bonds with  $\beta 3C^3$  and  $\gamma 2C^3$  mutants at the N-terminus. Molecular weight markers are indicated on the left. Monomer (m) and trimer (t) bands are marked with black triangles. The bands indicated with white triangles are thought to be non-specific bands.

Next, we examined whether the position of cysteine residues C<sup>1</sup> and C<sup>2</sup> at the N-terminus of LCC domains affects disulfide bond formation of heterotrimer. Cysteines C<sup>1</sup> and C<sup>2</sup> at the N-terminus of LCC domains usually have -C<sup>1</sup>-X-X-C<sup>2</sup>- motif, except α1 and α2 chains, which have -C<sup>1</sup>-X-X-X-C<sup>2</sup>- motif (24, 25). We made α3C\* mutant which has the -C<sup>1</sup>-X-X-A-C\*- motif instead of the -C<sup>1</sup>-X-X-C<sup>2</sup>- motif. Therefore, the position of cysteine C<sup>2</sup> was moved 1.5 angstrom far from C<sup>1</sup>. The results using α3C\* mutant showed that heterotrimer could form disulfide bonds normally by cysteine residues C<sup>1</sup> and C\* at the N-terminus of α3C\* LCC domains with β3C<sup>3</sup> and γ2C<sup>3</sup> mutants (Fig. 4B). This result suggested that interaction of α3 chains with β3-γ2 heterodimers at the N-terminus of LCC domains is not rigid but flexible.

### 3.4 Disulfide bond formation at the N-terminus of LCC domains with mutations of cysteines C<sup>1</sup> and C<sup>2</sup>

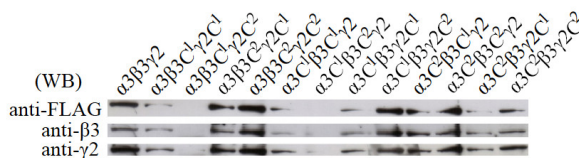
We co-expressed one single cysteine mutant among α3C<sup>1</sup>, α3C<sup>2</sup>, β3C<sup>1</sup>, β3C<sup>2</sup>, γ2C<sup>1</sup>, or γ2C<sup>2</sup> with two normal LCC domains of α3, β3, or γ2 chains to examine disulfide bond formation of heterotrimers. Using an anti-FLAG antibody for Western Blot, we did not detect any heterotrimer bands (Fig. 5). However, when using an anti-β3 antibody or an anti-γ2 antibody, we could detect very little amount of some heterotrimer bands with different size and migration (data not shown). By immunoprecipitation using anti-FLAG antibody, we could also detect the presence of β3 and γ2 chains using an anti-β3 antibody and an anti-γ2 antibody (data not shown). It means all the mutants interacted and assembled into dimers and trimers normally.



**Fig. 5.** Failure of disulfide bond formation by a single mutation of cysteines C<sup>1</sup> and C<sup>2</sup> at the N-terminus of LCC domains. Two normal LCC domains of α3, β3, and γ2 with one of the single cysteine mutants (α3C<sup>1</sup>, α3C<sup>2</sup>, β3C<sup>1</sup>, β3C<sup>2</sup>, γ2C<sup>1</sup>, and γ2C<sup>2</sup>) were separately co-

expressed and evaluated for heterotrimer formation by disulfide bonds at the N-termini. Samples were load into an 8% SDS-PAGE gel in non-reducing condition and detected by Western Blot with an anti-FLAG antibody. The results showed that without one cysteine C<sup>1</sup> or C<sup>2</sup> at the N-terminus of LCC domains of α3, β3, and γ2 chains, α3-β3-γ2 heterotrimers could not form. Molecular weight markers are indicated on the left. Monomer (m) and trimer (t) bands are indicated by black triangles. The bands indicated with white triangles are thought to be non-specific bands.

We then co-expressed two single cysteine mutants among α3C<sup>1</sup>, α3C<sup>2</sup>, β3C<sup>1</sup>, β3C<sup>2</sup>, γ2C<sup>1</sup>, or γ2C<sup>2</sup> with one normal LCC domain of α3, β3, or γ2 chains to check for the disulfide bond formation of heterotrimer. The results showed that some mutant combinations such as α3-β3C<sup>1</sup>-γ2C<sup>1</sup>, α3C<sup>1</sup>-β3C<sup>1</sup>-γ2, α3C<sup>1</sup>-β3-γ2C<sup>1</sup>, and α3C<sup>2</sup>-β3-γ2C<sup>1</sup> have weak efficiency of heterotrimer formation. Some other mutant combinations such as α3-β3C<sup>2</sup>-γ2C<sup>1</sup>, α3-β3C<sup>2</sup>-γ2C<sup>2</sup>, α3C<sup>1</sup>-β3-γ2C<sup>2</sup>, α3C<sup>2</sup>-β3C<sup>1</sup>-γ2, α3C<sup>2</sup>-β3C<sup>2</sup>-γ2, and α3C<sup>2</sup>-β3-γ2C<sup>2</sup> showed normal heterotrimer formation. But two special combinations of α3-β3C<sup>1</sup>-γ2C<sup>2</sup> and α3C<sup>1</sup>-β3C<sup>2</sup>-γ2 showed the failure of heterotrimer formation with disulfide bonds (Fig. 6). With three antibodies against α3, β3, and γ2 chains, we repeated this experiments several times and the results were the same. We could not identify which cysteine binds specifically to which cysteine at the N-terminus of LCC domains of LM-332. These results suggested that there is no specific disulfide bond pattern for cysteines at the N-terminus of LCC domains of LM-332.



**Fig. 6.** Disulfide bond formation of heterotrimers by two single mutations of cysteines C<sup>1</sup> and C<sup>2</sup> at the N-terminus of LCC domains. One normal LCC domain of α3, β3, or γ2 and two other single cysteine mutants among α3C<sup>1</sup>, α3C<sup>2</sup>, β3C<sup>1</sup>, β3C<sup>2</sup>, γ2C<sup>1</sup>, and γ2C<sup>2</sup> were co-expressed and examined for heterotrimer formation by disulfide bonds at the N-termini. Samples were loaded into an 8% SDS-PAGE gel in non-reducing conditions. Western Blot was first done with an anti-FLAG antibody to detect α3 chains, then the membrane was stripped and reprobred with an anti-β3 antibody to detect β3 chains, and finally stripped and reprobred again with anti-γ2 antibody to

detect  $\gamma 2$  chains. The results showed that LM heterotrimers formed with different efficiencies depending on single cysteine mutant combinations.

#### 4. Discussion

Cell-free translation system is a convenient system for recombinant protein expression and protein-protein interaction analysis. We succeeded in synthesizing heterotrimers of LCC domains of LM-332 in a cell-free translation system derived from *Spodoptera frugiperda* 21 (Sf21) insect cells (21-23). With the advantages of this system, we could analyze the disulfide bond formation of heterodimers and heterotrimers using many mutants. Our study focused on the disulfide bond formation of two cysteine residues  $C^1$  and  $C^2$  at the N-terminus and one cysteine residue  $C^3$  at the C-terminus of LCC domains, which involve in disulfide bond formation of LM heterodimer and heterotrimer.

Our previous data showed that when we expressed monomers of LCC domain of  $\alpha 3$ ,  $\beta 3$ , or  $\gamma 2$  chains, LCC domain of  $\beta 3$  or  $\gamma 2$  chains could form homodimers except  $\alpha 3$  chain, but not homotrimers. When we co-expressed LCC domain of  $\beta 3$  with  $\gamma 2$ , the main product was  $\beta 3$ - $\gamma 2$  heterodimers with a little amount of homodimers. This suggested that  $\beta 3$ - $\gamma 2$  heterodimers are more preferably formed comparing with  $\beta 3$ - $\beta 3$  and  $\gamma 2$ - $\gamma 2$  homodimers. Interchain hydrophobic interactions and ionic interactions of  $\alpha$ -helix coiled-coil domains drive the assembly of  $\beta 3$  and  $\gamma 2$  chains and that supports the disulfide bond formation by cysteines  $C^3$  at the C-termini. We did not find any trimers such as  $\beta 3$ - $\beta 3$ - $\gamma 2$ ,  $\beta 3$ - $\beta 3$ - $\beta 3$ ,  $\gamma 2$ - $\gamma 2$ - $\beta 3$ , and  $\gamma 2$ - $\gamma 2$ - $\gamma 2$  when we co-expressed LCC domains of  $\beta 3$  with  $\gamma 2$ . In this study, we mutated cysteines  $C^3$  at the C-termini of LCC domains of  $\beta 3$  and  $\gamma 2$  chains into alanines, we found that  $\beta 3C^3$  and  $\gamma 2C^3$  mutants could not form disulfide bonds by cysteines  $C^1$  and  $C^2$  at the N-termini. This result suggested that interchain interactions of LCC domains of  $\beta 3$  and  $\gamma 2$  at the N-terminus were not strong enough or incomplete to support the disulfide bond formation. Therefore, interchain hydrophobic interactions and ionic interactions at the C-terminus of LCC domains will drive the chain selectivity of LM  $\beta$  and  $\gamma$  chains. Then, cysteine  $C^3$  as a first lock will fix all preferably formed  $\beta$ - $\gamma$  heterodimers by a disulfide bond at the C-terminus of LCC domains.

When  $\alpha 3$  chain interacts with  $\beta 3$ - $\gamma 2$  heterodimer, the interaction of three chains at the N-terminus of LCC domains can make their cysteine residues come closer to each other and that interaction supports better for disulfide bond formation. The result using  $\alpha 3C^*$  mutant, which has the -C-X-X-X-C- motif instead of the -C-X-X-C- motif showed that the  $\alpha 3C^*$  mutant could form disulfide bonds normally with the  $\beta 3C^3$  and  $\gamma 2C^3$

mutants. This result suggested that interaction of  $\alpha 3$  chains with  $\beta 3$ - $\gamma 2$  heterodimers at the N-terminus of LCC domains are flexible with more than one interacting site. So the existence of LM heterotrimers that do not have disulfide bonds at the N-terminus of LCC domains could be because  $\alpha$  chains do not interact with  $\beta$ - $\gamma$  heterodimers at the site suitable for disulfide bond formation (12). From this result we also suggest that  $\beta 3$ - $\gamma 2$  heterodimer may be able to form disulfide bonds *in vitro* with all other LM  $\alpha$  chains by cysteines  $C^1$  and  $C^2$  at the N-terminus of LCC domains. Furthermore, to the best of our knowledge, this study is the first to show that without a disulfide bond at the C-termini of coiled-coil domain of  $\beta 3$ - $\gamma 2$  heterodimers, heterotrimers could be formed by disulfide bonds at the N-termini of coiled-coil domains. By using computer programs to analyze coiled-coil structure of all human and mouse LCC domains, Zimmerman and Blanco also concluded that it is possible for all LM  $\alpha$ ,  $\beta$ , and  $\gamma$  chains to form other non-natural disulfide-bonded heterotrimers out of 15 well-known natural LMs *in vitro* (26).

Our data showed that cysteines  $C^1$  and  $C^2$  at the N-termini of LCC domains are necessary for disulfide bond formation of heterotrimers. We created six single cysteine mutants including  $\alpha 3C^1$ ,  $\alpha 3C^2$ ,  $\beta 3C^1$ ,  $\beta 3C^2$ ,  $\gamma 2C^1$ , and  $\gamma 2C^2$ , and co-expressed these mutants with normal LCC domains to analyze which cysteine binds specifically to which cysteine at the N-terminus of LCC domains of LM-332. When we co-expressed two normal LCC domains with one single cysteine mutant, disulfide bonds could not form at the N-terminus of LCC domains. But when we co-expressed one normal LCC domains with two single cysteine mutants, disulfide bonds could form again with different efficiencies depending on mutant combinations. Furthermore, when we co-expressed three single cysteine mutants, heterotrimer could not form again (data not shown). These results suggested that the disulfide bond formation by cysteine residues  $C^1$  and  $C^2$  at the N-terminus of LCC domains of LM-332 are not fixed but random with different efficiencies depending on which cysteine binds to which cysteine. There should have other factors that interfere with or involve in the disulfide bond formation by binding to cysteine residues of LM chains. These factors could be any molecular chaperones involving in interchain and intrachain disulfide bond formation of proteins (27). Further research is needed to understand the mechanism of disulfide bond formation of LM heterotrimers inside the cell.

In conclusion, we provided new information about disulfide bond formation of LM heterodimers and heterotrimers. First, LM  $\alpha$ ,  $\beta$ , and  $\gamma$  chains are translated as monomers. Then,  $\beta$  and  $\gamma$  chains assemble to form  $\beta$ - $\gamma$  heterodimers with a disulfide bond at the C-terminus of

LCC domains. Next,  $\alpha$  chain assembles with  $\beta$ - $\gamma$  heterodimers. When  $\alpha$  chains interact with  $\beta$ - $\gamma$  heterodimers at suitable sites, disulfide bonds will form randomly at the N-terminus of LCC domains. Disulfide bond formation of heterotrimers at the N-termini does not depend on the disulfide bond formation at the C-termini of coiled-coil domains. We suggest that interactions at the N-terminus of LCC domains are important for disulfide bond formation of LM heterotrimers.

## ACKNOWLEDGEMENT

This study was supported by a Grant-in-aid (no. 18108003) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. HPP was supported by the Iwatani Naoji Foundation. We would like to sincerely thank Assoc. Prof. Tatsuhiko Kadowaki for his contribution in cloning laminin332 cDNA for this research.

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