Distinct roles of mouse laminin β1 long arm domains for α1β1γ1 trimer formation

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Abstract **Mouse embryonal carcinoma F9 cells expressing partial mouse laminin β1 covering either the C-terminal end (Δβ1S) or the whole (Δβ1L) of the long arm were established to study the assembly and interchain disulfide-bonding of β1 to endogenous laminin α1 and γ1. Both Δβ1S and Δβ1L were disulfide-bonded to γ1 but only Δβ1Lγ1 dimer formed a disulfide-bonded α1Δβ1Lγ1 trimer which was actively secreted into the medium. Meanwhile, in the cells producing Δβ1Sγ1 dimer, the level of endogenous α1β1γ1 was reduced but the level of monomeric α1 was increased, suggesting that α1 was recruited to trimer formation with the Δβ1Sγ1 dimer without disulfide-bonding. This shows that the Δβ1Sγ1 dimer can associate with α1 but not support the disulfide-bonding at the N-terminus of the long arm of α1. While control cells secrete neither monomeric α1 nor the β1γ1 dimer into the medium, the Δβ1Sγ1 producing cells probably do as α1Δβ1γ1 trimer. We thus propose that the N- and C-termini of the long arm of laminin β1 have distinct roles for trimer formation.**

Key words: Laminin; Heptad repeat; Disulfide-bonding

1. Introduction

Laminins constitute a family of basement membrane glycoproteins which affect tissue morphogenesis by their effects on proliferation, migration and differentiation of various types of cells [1–4]. The best studied mouse Engelbreth Holm Swarm (EHS) tumor isoform (laminin-1) is composed of α1 (400 kDa), β1 (220 kDa) and γ1 (210 kDa) chains, 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] of laminins have identified the long arm to be the site of chain assembly. It has many repeats of a heptad motif with hydrophobic residues at the first and fourth positions and charged residues 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 the N- and C-termini of the long arm while α1 is disulfide-bonded to β1 and γ1 only at the N-terminus of the long arm (Fig. 1, left panel). Since only αβγ trimers with the same N- to C-terminus orientation are selectively formed, intracellular assembly of laminin chains is a highly controlled process.

In experiments on de- and re-naturing proteolytic fragments of laminin 1, Engel and coworkers showed that E8 fragments of three chains, which correspond to the C-terminal end of the long arm, mimic normal intracellular assembly [13,14]. By preparing recombinant or synthetic peptides comprising mouse α1, β1 and γ1 partial sequences, Yamada and coworkers [15–18] showed that chain selection is controlled by defined sites at the C-terminal end of the long arm. Despite these in vitro data, the intracellular process of chain assembly is not well analyzed. Especially, not much is known about the interchain disulfide-bond formation between laminin chains.

In this study, we expressed mouse β1 sequences covering either the C-terminal end or the whole of the long arm in mouse embryonal carcinoma F9 cells and detected disulfide-bond formation between endogenous γ1 and the ectopic β1 mutant. Endogenous α1 was assembled to this hybrid β1γ1 dimer to form a α1β1γ1 trimer but it was disulfide-bonded only when the β1 sequence covered the whole long arm. While control cells secrete neither monomeric α1 nor β1γ1 dimer into the medium, cells producing the hybrid dimer secrete monomeric α1 probably by forming a α1β1γ1 trimer without disulfide-bonding between α1 and the hybrid β1γ1 dimer.

2. Materials and methods

2.1. Plasmid construction

Fig. 1 shows a simplified structure of laminin-1 and the domains of mouse laminin β1 expressed in F9 cells. The plasmids are based on pEFneoER (provided by Dr. A. Yoshimura) [19], which has erythropoietin receptor (ER) cDNA under the control of the elongation factor (EF) 1α promoter. The signal sequence of ER was used to insert the β1 sequence into the endoplasmic reticulum. For the construction of pEFΔβ1S, a cDNA fragment corresponding to the β1 sequence [10] of amino acid residues 1540–1765 was prepared with the polymerase chain reaction (PCR) using pLAM [20] as a template and two primers, 5′-GCTGGATCCGCTTGCAGCAGAGTGCAGCTGA-3′ and 5′-CGCGAATTCGCTAAGCAGGTGCTGTAAAACCG-3′. The ER cDNA fragment in pEFneoER was replaced by this fragment at *Bam*HI and *Eco*RI sites. For the construction of pEFΔβ1L containing the β1 sequence of amino acid residues 953–1765, a *Hinc*II-*Cla*I fragment of pLAM was inserted between the *Sma*I and *Cla*I sites of pEFΔβ1S.

2.2. Cloning of F9 cells expressing partial β1 sequences

Embryonal carcinoma F9 cells were maintained as stem cells [21] and transfected with 20 μg of plasmid DNA by calcium phosphate coprecipitation [22]. The *neo*-resistant clones were selected on a medium containing 0.25 mg/ml of G418. More than 10 Δβ1L-expressing cell lines were established and all gave the same results. However, only one Δβ1S-expressing cell line was available in this study.

2.3. Metabolic labeling of the cells and immunoprecipitation

The F9 cells in 35 mm dishes were labeled with 600 μl of methionine-free Eagle's minimal essential medium (Sigma) containing 0.3 mCi/ml Tran^{35}S label (ICN Radiochemical Inc.) for 4 h at 37°C. After removing the medium, the cells were lysed by adding 1000 μl of a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 5 mM EDTA, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1 mM phenylmethylsulfonyl fluoride (PMSF). Aliquots of cell lysate corresponding to 1.5×10^7 cpm of incorporated radioactivity into cellular

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proteins were diluted to 500 μl with the same buffer, and laminin chains were immunoprecipitated overnight at 4°C by mixing 1 μl of antiserum against mouse EHS laminin [23,24]. This antiserum immunoprecipitates α1β1γ1 trimers from various origins and gives stronger signals of β1 and γ1 relative to α1 in the immunoblotting of mouse laminin-1. Aliquots of labeled medium corresponding to the same size of culture were treated as above for the immunoprecipitation of secreted laminin chains.

2.4. SDS-gel electrophoresis

SDS-gel electrophoresis was carried out as described [23,24]. For one-dimensional electrophoresis under non-reducing condition, 3–10% (w/v) acrylamide was used. In two-dimensional electrophoresis, 4% gel was used for the first dimension under non-reducing condition and 3–10% gel was used for the second dimension under reducing condition. 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 and discussion

Our previous analyses of laminin assembly in mouse embryonal carcinoma F9 cells [25], mouse 3T3-L1 adipocytes [23] and bovine aortic endothelial cells [24] showed that β1 and γ1 are first disulfide-bonded through one pair of cysteines at the N- and C-termini of the long arm. Then α1 (or its homologue) associates to β1γ1 dimer and is disulfide-bonded to β1 and γ1 through two pairs of cysteines at the N-terminus of the long arm. The left panel of Fig. 1 is a simplified model of the structure of laminin-1 to show where these cysteine pairs are. The process of this intracellular chain assembly can be studied by metabolic labeling of the cells with [^{35}S]methionine/cysteine followed by immunoprecipitation of the cell lysate with anti-laminin antiserum and SDS-gel electrophoresis of the immunoprecipitates under non-reducing condition where inter-chain disulfide-bonds are preserved. As shown in Fig. 2A, α1β1γ1 trimer and β1γ1 dimer are detected in addition to monomeric α1, β1 and γ1 in control cells. Chain composition of the trimer and dimer can be confirmed by two-dimensional SDS-gel electrophoresis shown in Fig. 3, in which non-reducing electrophoresis in the first dimension (left to right) is 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. Since 4% and 3–10% acrylamide gels were used in Fig. 3 for the first and the second dimension, respectively, monomeric proteins smaller than 150 kDa were not separated in the first dimension and came to the vertical line at the right lower half of the gel. Many weak bands with migration faster than 200 kDa are precipitated from the cell lysates (in Fig. 2A). Since none of them has the reported or calculated molecular mass of endogenous or exogenous laminin chains, and most of them migrate as monomers in the two-dimensional electrophoresis (Fig. 3), we concluded they were precipitated non-specifically. To study the behavior of exogenous β1 fragments, we focused on bands specifically appeared in transgenic cells.

In the cells expressing Δβ1S or Δβ1L, exogenous β1 was recruited to the formation of a hybrid dimer and the band corresponding to the Δβ1Sγ1 or Δβ1Lγ1 dimer is detected under non-reducing condition (Fig. 2A, lanes Δβ1S and Δβ1L). Two-dimensional gel electrophoresis in Fig. 3 confirms that endogenous γ1 was selected by exogenous β1 for the formation of disulfide-bonded hybrid dimers of Δβ1Sγ1 or Δβ1Lγ1. In the cells expressing Δβ1L, the exogenous β1 was further recruited to the formation of an α1Δβ1Lγ1 trimer (Figs. 2 and 3), showing that the β1 sequence covering the

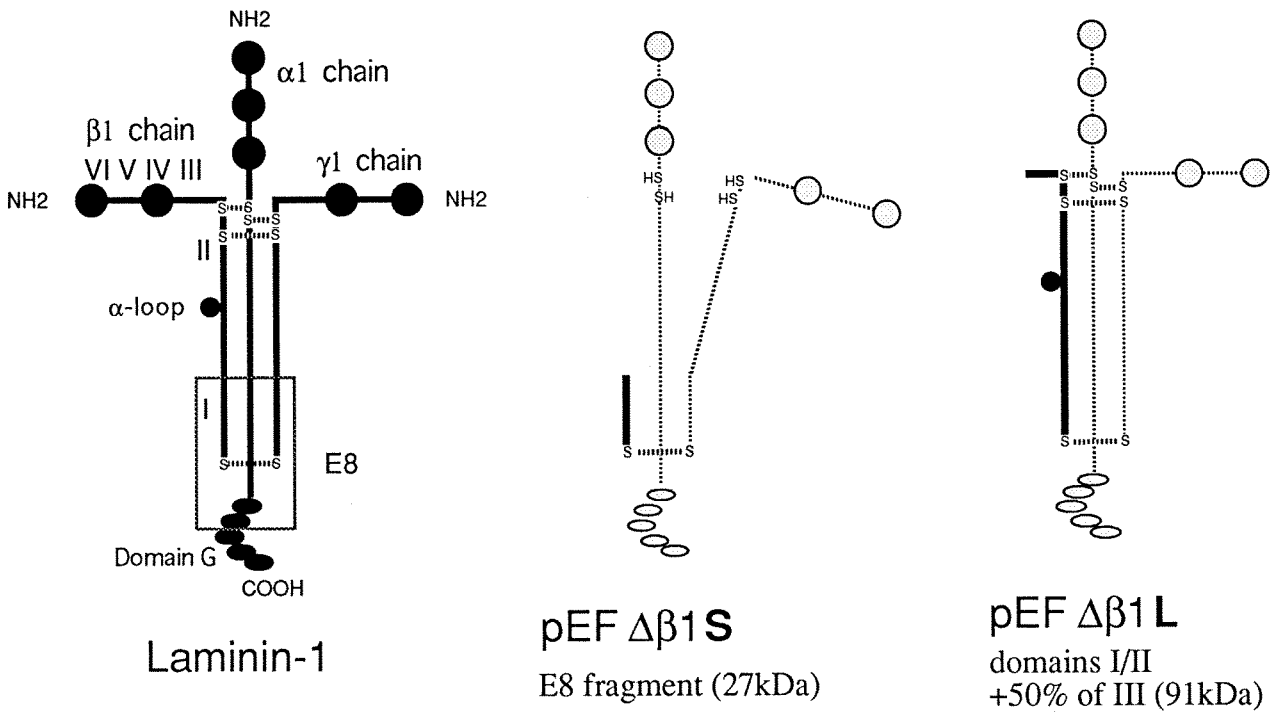

Fig. 1. Plasmids encoding the long arm domain of the mouse β1 chain. Simplified mouse laminin-1 structure, its major domains and cysteine pairs for interchain disulfide-bonding (S) are shown on the left. The long arm sequences encoded by pEFΔβ1S and pEFΔβ1L are shown by thick lines in the middle and on the right, respectively. The domains encoded by each plasmid are briefly explained together with the calculated molecular mass.

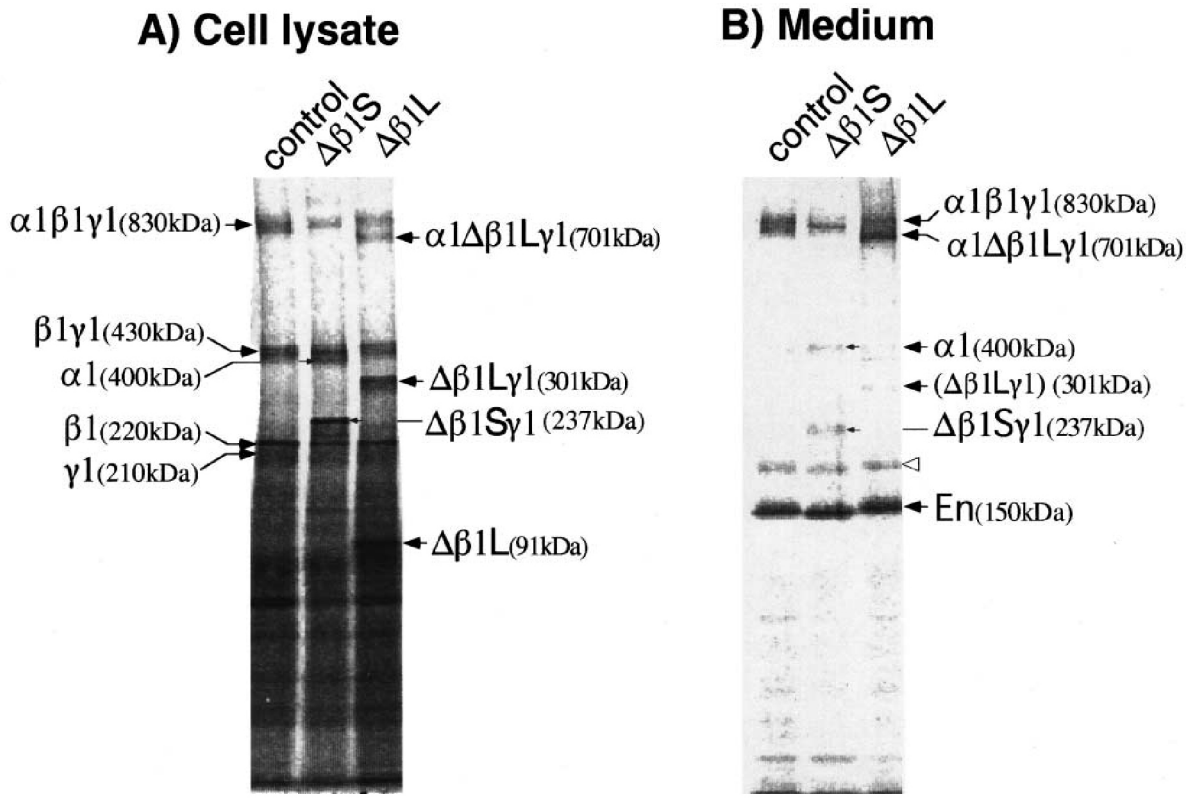

Fig. 2. Non-reducing SDS-gel electrophoresis of immunoprecipitates with anti-mouse laminin-1 antibody from F9 cells expressing partial mouse laminin β1 chain. F9 cells expressing none (control), Δβ1S or Δβ1L were labeled with [^{35}S]methionine/cysteine, the cell lysate and medium were immunoprecipitated with anti-mouse laminin-1 antiserum. Immunoprecipitates were separated by SDS-gel electrophoresis under non-reducing condition. Identification of laminin bands is indicated by arrows together with their calculated molecular mass in parentheses. 'En' indicates entactin/nidogen (150 kDa) which has strong affinity to the short arm of γ1 in the α1β1γ1 trimer. The band indicated by the open arrowhead is an unidentified protein immunoprecipitated even from the control cells.

whole long arm can support the association of endogenous α1 and its disulfide-bonding to the Δβ1Lγ1 dimer. As endogenous α1β1γ1 trimer, the α1Δβ1Lγ1 trimer was actively secreted into the medium (Fig. 2B, lane Δβ1L). In contrast, the C-terminal long arm sequence of β1 corresponding to the β1 segment of fragment E8 (Fig. 1) could not support the disulfide-bonding

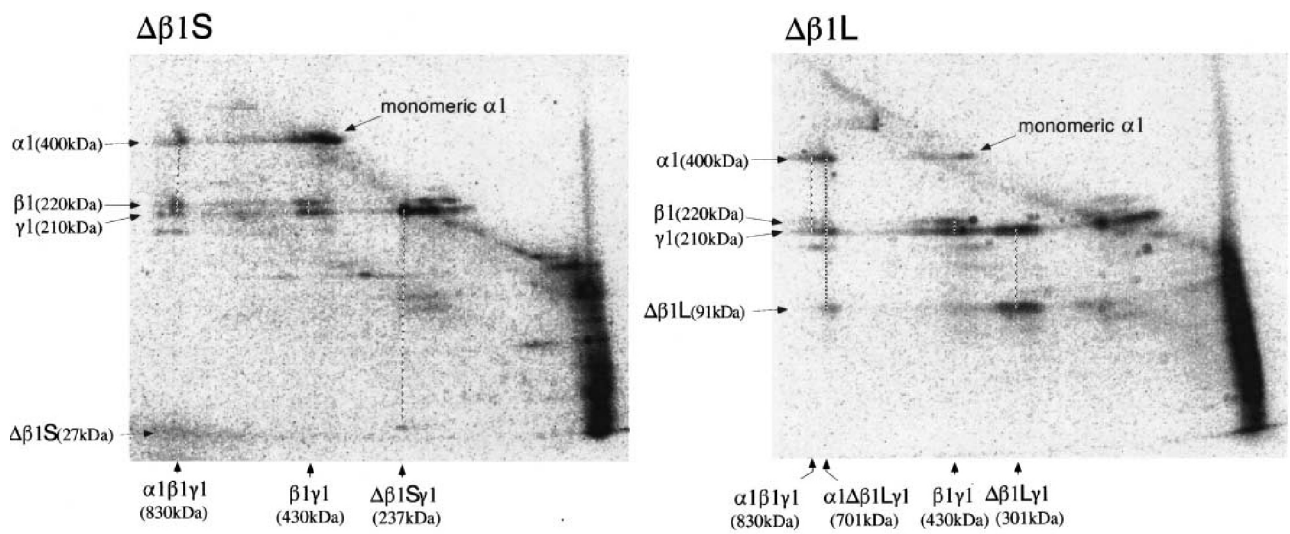

Fig. 3. Two-dimensional electrophoresis of immunoprecipitates with anti-mouse laminin-1 antibody from F9 cells expressing partial mouse laminin β1 chain. Immunoprecipitates from cell lysate prepared as in Fig. 2 were separated by SDS-gel electrophoresis under non-reducing condition in the first dimension (from left to right) followed by reducing condition in the second dimension (from top to bottom). Migration positions of disulfide-bonded complexes in the first dimension and monomers in the second dimension are indicated at the bottom and left margins of each panel, respectively, together with their calculated molecular mass in parentheses. Vertical dotted lines show proteins which are assumed to be disulfide-bonded to each other.

of α1 to the Δβ1Sγ1 dimer and no band of the hybrid trimer is detected in the cells expressing Δβ1S (Figs. 2 and 3). This suggests that a pair of cysteines for interchain disulfide-bonding between α1 and γ1 is not well oriented without the extension of coiled-coil formation between β1 and γ1 to the N-terminal end of the long arm (Fig. 1, middle panel).

Despite the failure of this disulfide-bond formation, however, the following observations from Figs. 2 and 3 suggest that the Δβ1Sγ1 dimer can associate with α1 to form the α1Δβ1Sγ1 trimer. First, the level of endogenous α1β1γ1 trimer is reduced both in the cell lysate and in the medium of Δβ1S-expressing cells when compared with the level in the control cells (Fig. 2). This suggests that one component of the α1β1γ1 trimer is depleted due to the expression of Δβ1S. Second, the level of monomeric α1 in the cell lysate is increased in Δβ1S-expressing cells (Fig. 2A). This fact was not clear in the non-reducing electrophoresis in Fig. 2 due to the poor separation of α1 from β1γ1, but the two-dimensional electrophoresis in Fig. 3 undoubtedly shows that monomeric α1 is specifically increased relative to other spots. SDS-electrophoresis of the same cell lysate under reducing condition showed that the total amount of α1 remains unchanged regardless of the type of exogenous β1 expressed (result not shown). This result is consistent with the model that a part of α1 is assembled to the α1Δβ1Sγ1 trimer without disulfide-bonding to the Δβ1Sγ1 dimer but migrated as a monomer in the electrophoresis under reducing condition. Third, a small amount of monomeric α1 was secreted into the medium by Δβ1S-expressing cells together with the Δβ1Sγ1 dimer (Fig. 2B). In our previous studies [23–25], endogenous laminin chains were allowed to leave the endoplasmic reticulum only after the assembly into disulfide-bonded trimers. Neither monomeric chains nor β1γ1 dimers were transported to the secretory pathway as in the control cells (Fig. 2B). These results suggest that there is a selection mechanism which allows only the αβγ trimers to leave the endoplasmic reticulum. Since monomeric α1 together with the Δβ1Sγ1 dimer is secreted by Δβ1S-expressing cells, the C-terminal sequence of β1 corresponding to the β1 segment of fragment E8 is enough to assemble the trimer which can pass through this selection mechanism. Immunoprecipitation of the Δβ1Sγ1 dimer with α1-specific antibody might provide solid evidence for trimer formation but an appropriate antibody is not available to us. Although the band was too weak to be identified, an extremely small amount of Δβ1Lγ1 dimer appeared to be secreted also from the cells expressing Δβ1L probably together with monomeric α1 (Fig. 2B). This suggests that a part of the Δβ1Lγ1 dimer failed to be disulfide-bonded to α1 but secreted as α1Δβ1Lγ1 trimer as well.

In summary, we demonstrated that the mouse β1 long arm sequence has distinct domains for trimer assembly. Consistent with the results of in vitro experiments, we showed here that the C-terminal end of β1 was also enough for the in vivo chain selective assembly of the α1β1γ1 trimer. Our in vivo experiment made it possible to analyze the function of the whole long arm for the first time and demonstrated that extension of the coiled-coil formation between β1 and γ1 to the N-terminal end is essential for α1 to orient itself for the interchain disulfide-bonding to β1 and γ1. Since α1, β1 and γ1 short arms are combined by three pairs of cysteines at the N-terminal end of the long arm (Fig. 1) and the correct orientation of the short arms is important for the interaction of laminin with cells and other basement membrane components, we propose that the N-terminal domain of the β1 long arm is essential for the organization of functional laminin structure.

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