Potential molecular chaperones involved in laminin chain assembly

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Abstract

To explore potential molecular chaperones involved in the intracellular assembly of laminin chains, bovine aortic endothelial cells were treated with a thiol cleavable divalent cross-linking reagent, dithio-bis-(succinimidylpropionate), and cellular proteins cross-linked to laminin chains were co-immunoprecipitated with anti-laminin antiserum. Sodium dodecylsulfate (SDS) gel electrophoresis of the precipitate under reducing condition showed polypeptides with estimated sizes of 80, 60 and 50 kDa together with laminin chains. Two dimensional electrophoresis, in which non-reducing and reducing SDS electrophoresis were combined, suggested that many molecules of these polypeptides were cross-linked to each laminin chain. Sepharose CL-4B beads conjugated with E8 fragment of mouse laminin-1 was prepared. Affinity chromatography with the beads of microsomal proteins from rat liver showed that Bip and HSP70 associated to laminin chains and dissociated upon ATP hydrolysis. Protein-disulfide isomerase also showed affinity to the column. GRP94 and calnexin showed strong affinity and were washed out only with a detergent solution. Thus, many molecular chaperones are suggested to be involved in the intracellular assembly of laminin chains.

Abbreviations: BAEC, bovine aortic endothelial cells; Bip, immunoglobulin-binding protein; DMEM, Dulbecco's modified Eagle's medium; DSP, dithio-bis-(succinimidylpropionate); ECL, enhanced chemiluminescence; EHS, Engelbreth-Horm-Swarm; ER, endoplasmic reticulum; GRP, glucose response protein; HSP, heat shock protein; NEM, N-ethylmaleimide; PBS, phosphate buffered saline; PDI, protein-disulfide isomerase; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecylsulfate

Introduction

Laminins are a family of basement membrane glycoproteins in which α , β and γ chain are assembled and disulfide-bonded in a cross shaped structure with three short arms and one rod-like long arm (Engel *et al.*, 1981; Engel, 1993; Burgeson *et al.*, 1994). The long arm is the site of chain assembly (Paulsson *et al.*, 1985) and has many repeats of the heptad motif in which hydrophobic residues locate in the first and fourth positions and charged residues in the fifth and seventh positions (Beck *et al.*, 1993). They form a hydrophobic surface along α -helix with ionic edges at both sides. It is suggested that interchain hydrophobic interactions at this surface drive the chain assem-

bly and ionic interactions at the edges determine the chain selectivity (Beck *et al.*, 1993). Many variants of laminin chains have been cloned. From mouse, $\alpha 1$ (Sasaki *et al.*, 1988), $\alpha 2$ (Bernier *et al.*, 1995), $\alpha 3$ (Galiano *et al.*, 1995), $\alpha 5$ (Miner *et al.*, 1995), $\beta 1$ (Sasaki *et al.*, 1987), $\beta 2$ (Durkin *et al.*, 1996), $\beta 3$ (Utani *et al.*, 1995), $\gamma 1$ (Sasaki and Yamada, 1987) and $\gamma 2$ (Sugiyama *et al.*, 1995) have been cloned. From human, $\alpha 1$ (Nissinen *et al.*, 1991), $\alpha 2$ (Vuolteenaho *et al.*, 1994; Zhang *et al.*, 1996), $\alpha 3$ (Ryan *et al.*, 1994; Vidal *et al.*, 1995), $\alpha 4$ (Iivanainen *et al.*, 1995; Richards *et al.*, 1996), $\beta 1$ (Pikkarainen *et al.*, 1987), $\beta 2$ (Wewer *et al.*, 1994; Iivanainen *et al.*, 1994), $\beta 3$ (Gerecke *et al.*, 1994), $\gamma 1$ (Pikkarainen *et al.*, 1988) and $\gamma 2$ (Kallunki *et al.*, 1992, and Vailly *et al.*, 1994)

have been cloned. Amino acid sequences of the long arms are varied among α , β and γ chains from different animal species but they all have well aligned heptad motif. It implies that all laminin chains share a common mechanism of the assembly. Chain replacement during assembly creates many laminin isoforms. For example, replacement of $\alpha 1$ of $\alpha 1\beta 1\gamma 1$ trimer (laminin 1) by $\alpha 2$ produces muscle membrane laminin (laminin 2) (Ehrig *et al.*, 1990; Marinkovich *et al.*, 1992; Vuolteenaho *et al.*, 1994) while replacement of $\beta 1$ by $\beta 2$ results in a synaptic laminin (laminin 3) (Hunter *et al.*, 1989). Similar replacement has been suggested for the formation of kalinin (laminin 5) and K-laminin (laminin 6) which have the chain compositions of $\alpha 3\beta 3\gamma 2$ and $\alpha 3\beta 1\gamma 1$, respectively (Roussle *et al.*, 1991; Kallunki *et al.*, 1992). Since only $\alpha\beta\gamma$ trimers in the same N- to C-terminus orientation is selectively formed, the intracellular assembly of laminin chains is a highly controlled process.

Our analyses on various mammalian cell lines showed that β and γ chains first form disulfide-bonded dimer and α chain is disulfide-bonded to $\beta\gamma$ dimer to form $\alpha\beta\gamma$ trimer (Morita *et al.*, 1985; Aratani and Kitagawa, 1988; Tokida *et al.*, 1990). Analysis on a *Drosophila* cell line showed essentially the same process for insect laminin chain assembly. Advantage of the chain specific antibodies against *Drosophila* laminin revealed that β and γ chains form a stable dimer prior to their disulfide-bonding and that α chain associates to only the disulfide-bonded $\beta\gamma$ dimer (Kumagai *et al.*, 1997). We also found that laminin chains are allowed to leave from ER for secretion only after the assembly into trimer (Morita *et al.*, 1985; Aratani and Kitagawa, 1988; Tokida *et al.*, 1990). The same was true for *Drosophila* laminin although *Drosophila* α chain can be secreted either disulfide-bonded or not bonded to the disulfide bonded $\beta\gamma$ dimer (Kumagai *et al.*, 1997). Expression of truncated mouse β chain ($\Delta\beta$) in mouse embryonal carcinoma F9 cells resulted in the assembly of $\alpha\Delta\beta\gamma$ trimer with endogenous α and γ chains (Niimi and Kitagawa 1997). In this trimer, $\Delta\beta$ and γ chains were disulfide-bonded but α chain was not bonded to $\Delta\beta\gamma$ dimer. Nevertheless, α chain was allowed to be secreted only after the trimer formation. These results altogether suggest a selection mechanism which allow only the trimers to be secreted.

Taking two independent approach, we here explore potential molecular chaperones responsible to such selection mechanism. In the first approach, we took advantage of DSP which can cross-link intracellularly associated molecules by thiol cleavable bridge. After

treating laminin producing cells with DSP, the cell lysate was immunoprecipitated with anti-laminin. SDS gel electrophoresis of the precipitate showed polypeptides with estimated sizes of 80, 60 and 50 kDa together with laminin chains. In the second approach, we developed an affinity chromatography to detect microsomal proteins which bind mouse laminin-1 ($\alpha 1\beta 1\gamma 1$). The column showed that GRP94, calnexin, Bip, HSP70 and PDI have the affinity to laminin chains.

Material and methods

Cell culture

BAEC isolated as described by Gosdarowicz (1976) was maintained in DMEM containing 10% fetal calf serum and antibiotics.

Antibodies

Anti-EHS laminin rabbit antiserum was prepared as described (Morita *et al.*, 1985; Aratani *et al.*, 1988; Tokida *et al.*, 1990). Anti-chicken GRP94 rat monoclonal IgG, anti-water mold (*Achlya ambisexualis*) HSP90 rat monoclonal IgG, anti-human Bip C-terminal peptide rabbit IgG, and anti-human HSP70 mouse monoclonal IgG were purchased from Stress-Gen. Anti-rat PDI monoclonal IgG was from Affinity BioReagent Inc. Anti-calnexin rabbit serum (Wada *et al.*, 1994) was kindly provided by Dr. Ikuo Wada.

Labeling cells with [^{35}S]-methionine/cysteine and cross-linking with DSP

Confluent culture of BAEC in 60 mm cell culture dishes were labeled with 0.5 mCi/ml of Tran[^{35}S] label (ICN) for 4 h at 37 °C. The cultures were washed once with PBS, trypsinized and suspended in 100 ml solution of 2 mM DSP in PBS. After incubation on ice for 30 min, the cultures were rinsed with 2 mM glycine in PBS to block the cross-linking reaction and then washed with PBS (Nagai *et al.*, 1992).

Immunoprecipitation and SDS gel electrophoresis

Immunoprecipitation of laminin-related polypeptides and SDS gel electrophoresis were carried out as described previously (Aratani and Kitagawa, 1988).

Sepharose CL-4B beads conjugated with laminin E8 fragment

Laminin E8 fragment was prepared as described by Paulsson *et al.* (1987). Briefly, EHS tumor was homogenized with buffer A (Tris-HCl pH 7.4 and 0.15 M NaCl) containing 2 mM PMSF and 2 mM NEM. Pellet was extracted for 16 h at 4 °C with buffer B containing 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 10 mM EDTA, 1 mM PMSF and 1 mM NEM. The extract was separated by gel filtration (Sephacrose CL-6B, Pharmacia) with buffer B. The first peak containing laminin and nidogen was dialyzed against buffer A containing 2 mM EDTA and 0.5 mM NEM, then digested with 0.5 mg/mg protein of elastase (Elastin Products Co. Inc.) for 24 h at 37 °C. The digest was separated by gel filtration (Sephacrose CL-6B). The third peak was used as E8 fragment fraction. E8 fragment-conjugated beads were prepared using CNBr-activated Sepharose CL-4B (Pharmacia). Five mg of E8 fragment was immobilized to 1 ml of beads.

Preparation of microsome fraction from rat liver

Rat liver was homogenized with Dounce homogenizer in buffer C (20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 250 mM sucrose). Debris was removed twice by centrifugation at $20\,000 \times g$ for 30 min. The supernatant was further centrifuged at $100\,000 \times g$ for 1 h. The microsomal pellet was suspended in buffer C and stored at -80 °C.

Affinity chromatography and Western analysis

Affinity chromatography was done as described by Nigam *et al.* (1994). E8 fragment conjugated to Sepharose CL-4B was denatured and reduced by incubating the beads in 2 ml of 6 M urea and 1 M 2-mercaptoethanol solution for 30 min. Slurry of the beads of 0.1 ml packed volume were mixed with 0.5 mg rat microsomal proteins dissolved in 2 ml of 20 mM Tris-HCl pH 7.4, 75 mM NaCl, 0.125 M sucrose, 2.5 mM EDTA, 1 mM EGTA and 0.5% Triton X-100 and the bead suspension was transferred to a column. The column was sequentially washed with 1) 2 ml of a buffer containing 20 mM Tris-HCl pH 7.4, 2.5 mM EDTA and 1 mM EGTA, 2) 2 ml of a buffer containing 20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$, 3) 2 ml of a buffer containing 20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM ATP, 4) 2 ml of 0.1 M acetic acid, and 5) 2 ml of a

buffer containing 20 mM Tris-HCl pH 7.4, 1% Triton X-100 and 1 M NaCl. Eluted proteins were concentrated by precipitation with 10% trichloroacetic acid and separated by SDS gel electrophoresis under reducing condition using a 7% polyacrylamide gel. The proteins were transferred to Hybond ECL (Amersham) and analyzed using Amersham ECL Western blotting analysis system. To detect GRP94, anti-rat IgG mouse IgG (Jackson Laboratory) was reacted prior to the reaction with horse radish peroxidase-conjugated anti-mouse IgG.

Results

DSP cross-linking of cellular proteins to laminin chains in BAEC

To explore potential molecular chaperones involved in the laminin chain assembly, we took advantage of membrane permeable DSP which can cross-link cellular proteins *in vivo* by N-hydroxysuccinimide ester reaction and which is thiol cleavable bridge. Metabolically radiolabeled BAEC, a laminin producing cell line, were incubated with DSP and cell lysate was prepared for immunoprecipitation with anti-laminin antiserum. When immunoprecipitates were separated by SDS gel electrophoresis under non-reducing condition (Fig. 1A/NR), DSP cross-linking reduced monomeric $\alpha 1$, αx (a variant of α chain produced in BAEC having two intracellular forms of $\alpha x \alpha$ and $\alpha x \beta$ depending on different steps of N-glycan processing (Tokida *et al.*, 1990)), $\beta 1$ and $\gamma 1$, and $\beta 1 \gamma 1$ dimer compared to the immunoprecipitates from the control cell. Most of the radioactive proteins migrated as bands with extremely large sizes. By SDS gel electrophoresis under reducing condition (Fig. 1A/R), on the other hand, all laminin chains could be detected even after cross-linking. This suggests that laminin chains were cross-linked to cellular proteins with thiol cleavable bridge. In Fig. 1A, a 4% acrylamide gel was used in order to monitor the behavior of laminin chains having the sizes of 200–400 kDa and the smaller proteins cross-linked to laminin chains were not well separated. When the same sample was separated in a 8% acrylamide gel (Fig. 1B), three major bands with estimated sizes of 80, 60 and 50 kDa were detected together with laminin chains. Since emergence of these bands clearly depended on the DSP cross-linking, they are suggested to be located close to laminin chains. The amount of these polypeptides co-precipitated with laminin chains was too small

Figure 1. Analysis of the cellular proteins cross-linked to laminin chains with DSP. Metabolically radiolabeled BAEC were incubating on ice with (+) or without (-) 2 mM DSP for 30 μ M, and laminin in cell lysates were immunoprecipitated together with cross-linked proteins and separated by SDS gel electrophoresis in a 4% (panel A) or a 8% (panel B) gel under non-reducing (NR) or reducing (R) condition. The bands co-precipitated with laminin chains after cross-linking with DSP treatment are indicated with open arrowheads in panel B. Migration positions of molecular weight markers and laminin chains are indicated.

to be identified by Western analysis using antibodies against the general molecular chaperones such as chicken GRP94, water mold HSP90, chicken HSP47, human Bip, rat PDI and dog calnexin. Immunoprecipitation of the lysate from DSP cross-linked cells with either of these antibodies failed to show radiolabeled bands corresponding to laminin chains. Thus, identity of the 80, 60 and 50 kDa polypeptide is open to further characterization.

Figure 2 shows two-dimensional SDS gel electrophoresis of the same immunoprecipitates in which non-reducing electrophoresis from *left to right* was

followed by reducing electrophoresis from *top to bottom*. When immunoprecipitate from the control cells was separated by this electrophoresis (Fig. 2A), the monomeric polypeptides such as $\alpha 1$, $\beta 1$, αx and $\gamma 1$ migrate on the diagonal while the disulfide-bonded polypeptides such as $\beta 1 \gamma 1$ dimer, $\alpha x \beta 1 \gamma 1$ and $\alpha 1 \beta 1 \gamma 1$ trimers migrate to the area below the diagonal. DSP cross-linking markedly reduced monomeric laminin chains and $\beta 1 \gamma 1$ dimer which migrated slower under reducing condition probably due to thiol cleavable bridge to cellular proteins (Fig. 2B). Such cellular proteins were cleaved from laminin chains under

Figure 2. Non-reducing and reducing two-dimensional SDS gel electrophoresis of the cellular proteins cross-linked to laminin chains with DSP. Radiolabeled cell lysates from BAEC without (panel A) or with (panel B) incubation with 2 mM DSP were immunoprecipitated as in Fig. 1. The precipitates were separated by two-dimensional SDS gel electrophoresis from *left to right* under non-reducing conditions and from *top to bottom* under reducing conditions.

Figure 3. Molecular chaperones bound to E8 fragment of mouse laminin-1 conjugated to Sepharose CL-4B beads. E8 fragment of mouse laminin-1 was conjugated to Sepharose CL-4B. After denaturation and reduction of E8 fragment by incubating the beads in a solution containing urea and 2-mercaptoethanol, the beads were incubated with microsomal proteins from rat liver. The bead suspension was transferred to a column and sequentially eluted with 1) the buffer containing EDTA and EGTA, 2) the buffer containing Ca^{2+} and Mg^{2+} , 3) the buffer containing Ca^{2+} , Mg^{2+} and ATP, 4) acetic acid, and 5) the buffer containing Triton X-100 and NaCl. The eluted proteins were precipitated with 10% trichloroacetic acid, separated by SDS gel electrophoresis under reducing condition and detected by Western analysis using antibodies against GRP94, calnexin, Bip, HSP70 and PDI.

reducing condition and run off the bottom of gel. Horizontal trailing of the spots shows that each laminin chain is cross-linked to cellular proteins with various molar ratio. This suggests that more than one molecule of molecular chaperones associate to each laminin chain. Among three laminin chains, $\beta 1$ monomer is suggested to have the most rigid structure to guide the association of $\gamma 1$ chain to form $\beta 1\gamma 1$ dimer and then the most flexible $\alpha 1$ chain associates to $\beta 1\gamma 1$ dimer to form $\alpha 1\beta 1\gamma 1$ trimer (Nomizu *et al.*, 1996). Probably reflecting such structure of $\beta 1$ chain, it is worth to note that $\beta 1$ chain was the most inert in the cross-linking reaction.

Binding of Bip, HSP70, PDI, GRP94 and calnexin to laminin-beads

As another approach to the molecular chaperones, we developed affinity chromatography by preparing Sepharose CL-4B beads to which mouse laminin-1 ($\alpha 1\beta 1\gamma 1$) from EHS tumor was conjugated. Since whole laminin-1 molecule caused aggregation of the beads due to self-association at N-termini of the short arms (Yurchenco *et al.*, 1992; Yurchenco and Cheng, 1993), E8 fragment corresponding to C-termini of the long arm was conjugated. *In vitro* experiments have shown that E8 fragment contains the essential sequence of $\alpha 1$, $\beta 1$ and $\gamma 1$ for the assembly and can mimic the

Figure 4. Model for the role of molecular chaperones in the laminin chain assembly. The figure shows the top view of the long arm region of each laminin chain. The heptad repeat of $(abcdef)_n$ creates the surface composed of hydrophobic amino acids (closed circles) with charged amino acids (dotted circles) at both edges. The hydrophobic surface interacts with the molecular chaperones which retain the monomeric and dimeric laminin chain in the lumen of ER. See the text for more detail.

process of intracellular chain assembly (Hunter *et al.*, 1990 and 1992). As the source of molecular chaperones, rat liver microsomal fraction was applied to the columns of laminin-beads in a buffer containing

0.5% Triton X-100 for solubilization of microsomal proteins. The column were eluted with 1) the buffer containing EDTA and EGTA, 2) the buffer containing Ca^{2+} and Mg^{2+} , 3) the buffer containing Ca^{2+} ,

Mg²⁺ and ATP, 4) acetic acid, and 5) the buffer containing Triton X-100 and NaCl. As summarized in Fig. 3, Western analysis of the elute showed that Bip and HSP70 bound to laminin-beads and were eluted depending on their activity of ATP hydrolysis. GRP94 and calnexin also bound to the beads and were eluted only by the buffer containing 1% Triton X-100 and 1M NaCl. PDI, which is potentially responsible to the disulfide-bonding of laminin chains, seemed to interact with laminin chains in multiple mechanism. Despite of extensive washing of the beads with the buffer containing EDTA and EGTA, a considerable amount of PDI was eluted with the buffer containing Ca²⁺/Mg²⁺, and the buffer containing Triton X-100/NaCl gave another elution of PDI.

In the experiment shown in Fig. 3, E8 fragment conjugated to the beads was denatured and reduced prior to challenging the microsomal proteins. A parallel experiment without this treatment showed that this denaturation and reduction process did not affect the variety and the quantity of molecular chaperones having the affinity to the beads.

We also tried to detect the molecular chaperones in BAEC but amount of the microsomal fraction available from BAEC was limited to design this experiments. For embryonal carinoma F9, another laminin producing cell line (Morita *et al.*, 1985), weak signals in the Western analysis suggested similar behavior of Bip, HSP70 and PDI as for rat liver microsomal fraction (result not shown).

Discussion

For selective recognition of laminin chains, the interchain ionic and hydrophobic interaction created by the heptad repeats in the long arm region are crucial. This implies that the amino acids sequences of the long arm region can automatically control the laminin chain assembly. Since the process includes the step at which the hydrophobic surface of the monomeric and dimeric laminin chains is exposed (Fig. 4), however, we need to assume some molecular chaperones which can neutralize this hydrophobic surface and prevent the aggregation of laminin chains before the assembly. In addition, our analyses of the intracellular assembly in various cell lines showed that laminin chains are allowed to leave from ER for secretion only after the assembly into trimer (Morita *et al.*, 1985; Aratani and Kitagawa, 1988; Tokida *et al.*, 1990; Niimi and Kitagawa 1997; Kumagai *et al.*, 1997). This also suggests

important role of the molecular chaperones in selective transport of laminin chains to the secretion pathway. Our present study has demonstrated that many cellular proteins including general molecular chaperones like Bip, HSP70, GRP94, calnexin have affinity to laminin chains.

Figure 4 depicts our tentative scenario of the laminin chain assembly which can be summarized as the followings. 1) The long arm region of each laminin chain forms the structure of α -helix which creates a hydrophobic surface along the molecular α axis with charged amino acid residues line up at both edges. 2) Molecular chaperones like Bip and HSP70 are responsible to prevent the malfolding of α -helix in expense of ATP hydrolysis. 3) Many molecules of molecular chaperones associate to each molecule of laminin chain to keep the rod-like laminin chains soluble. 4) Since some of the molecular chaperones have the ER retention signal (for example KDEL), the monomeric laminin chains are withheld within lumen of ER. 5) β and γ chains associate to each other driven by the interchain hydrophobia and ionic interaction. 6) PDI catalyzes the interchain disulfide-bonding between β and γ chains. 7) Even after the formation of disulfide-bonded $\beta\gamma$ dimer, the hydrophobic surface remains to be neutralized by the molecular chaperones and they still arrest the dimer in ER. 8) α chain associates to $\beta\gamma$ dimer and neutralizes its own and the dimer's hydrophobic surface. 9) Since no more hydrophobic surface is remained after the trimer formation, the trimer is allowed to leave from ER for the secretion. 10) The disulfide-bonding between α chain and $\beta\gamma$ dimer is not the requisit of secretion but PDI, having affinity to laminin chains, catalyses this reaction.

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