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Post-translational assembly and glycosylation of laminin subunits in parietal endoderm-like F9 cells

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Non-reducing and reducing sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of laminin synthesized in parietal endoderm-like F9 cells demonstrated that only AB_1B_2 complex goes through intracellular traffic for oligosaccharide side-chain processing and secretion. Glycosylation was not necessary for subunit assembly. Assembly was suggested to proceed through B_1B_2 to AB_1B_2. Among the pools of monomer subunits, the B_2 pool was smallest.

Laminin (Timple *et al.*, 1979) is a major glycoprotein in basement membranes and is composed of at least three *N*-glycosylated polypeptides: subunit A (M_r 450000), B_1 (240000) and B_2 (230000) (Cooper *et al.*, 1981). Laminin is secreted from parietal endoderm cells in the mouse embryo (Hogan *et al.*, 1980), but a system of embryonic carcinoma F9 cells *in vitro* also secretes it when the cells are converted into parietal endoderm-like F9 cells by retinoic acid and cyclic AMP (Strickland *et al.*, 1980). By using various parietal endoderm-like cells, synthesis of laminin polypeptides and targeting them into the lumen of RER (Kurkinen *et al.*, 1983), addition and processing of oligosaccharide side chains (Cooper *et al.*, 1981), assembly of laminin subunits into complexes by disulphide-bonding (Cooper *et al.*, 1981) and intracellular transport from RER through Golgi cisternae to secretory granules (Laurie *et al.*, 1982) have been investigated. However, no information is available concerning the sequence of assembly of A, B_1, and B_2 into complexes and the interrelation between assembly and processing of oligosaccharide side chains. Because the cytological sites of these modifications are supposed to lie in the RER or in the proximal or distal Golgi cisternae, elucidation of the sequence of subunit assembly and processing of oligosaccharide side chains might provide important information about the interrelationship between RER and Golgi cisternae during processing and intracellular transport of laminin subunits.

In the present study the assembly and glycosylation of laminin subunits in parietal endoderm-like F9 cells have been analysed by two-dimensional SDS/polyacrylamide-gel electrophoresis, in which non-reducing and reducing electrophoresis are combined. Tunicamycin (Takatsuki *et al.*, 1971) has been used to study the role of oligosaccharide side chains in the assembly. Effects of blocking the exit of Golgi cisternae by monensin (Tartakoff, 1982) on assembly and glycosylation of laminin subunits were studied. These analyses provided several novel pieces of information concerning the mechanism of assembly and glycosylation of laminin subunits.

Experimental

Embryonal carcinoma F9 cells were maintained and differentiated essentially as described by Strickland *et al.* (1980). Parietal endoderm-like F9 cells were prepared by incubating cells with 0.1 μM-retinoic acid (Sigma) and 1 mM-dibutyryl cyclic AMP (Boehringer) for 6 days. Before labelling, cells were pretreated for 2 h with no addition, 1 μM-monensin (Calbiochem) or tunicamycin (1 μg/ml) (Seikagaku Kogyo Co., Tokyo, Japan). Labelling of cells was started by changing the medium to 30 μl of methionine-free Eagle's minimum essential medium (Nissui Seiyaku Co., Tokyo, Japan) containing 1 mCi of [^{35}S]methionine (Amersham)/ml at a final specific radioactivity of 125 Ci/mmol, and the same antibiotics at the same concentrations as during pretreatment. After labelling at 37°C for the indicated periods, media were

Abbreviations used: SDS, sodium dodecyl sulphate; RER, rough endoplasmic reticulum.

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collected and cells were lysed in the immunoprecipitation buffer containing 10mM-Tris/HCl, pH8.0, 2mM-EDTA, 1% Triton X-100, 0.1% SDS, 0.4M-NaCl, 1mM-phenylmethanesulphonyl fluoride and 50mM-iodoacetamide. Collected media were centrifuged to remove floating cells, and mixed with equal volumes of two-times-concentrated immunoprecipitation buffer. Trichloroacetic acid-insoluble radioactivity in the labelled cell lysates and media was determined by a filter-disc method (Roberts & Paterson, 1973).

Portions of labelled cell lysate and medium were adjusted to 300μl with the immunoprecipitation buffer and incubated for 1h with 50μl of 10% suspension of *Staphylococcus* cells (Pansorbin; Calbiochem) at room temperature. After centrifugation, the supernatant was incubated for 1h with 3μl of antiserum against laminin (Bethesda Research Laboratories) followed by the addition of 50μl of Pansorbin and a further 1h of incubation. The final pellet was washed three times with the immunoprecipitation buffer and extracted with SDS sample buffer, containing 6% (w/v) SDS, 0.133mM-Tris/HCl, pH6.8, 10% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol and 0.06% Bromophenol Blue, by heating at 90°C for 4min. Under non-reducing conditions, 2-mercaptoethanol was omitted from the SDS sample buffer. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970), with 3% (w/v) acrylamide for the stacking gel and 4% (w/v) acrylamide for the separating gel. Two-dimensional electrophoresis was carried out by the method of Dulis *et al.* (1982). The first-dimension non-reducing SDS/gel electrophoresis was done with 4% acrylamide gels in tubes. These gels were then incubated in SDS sample buffer for 15min and applied to slab gels of 4% acrylamide. After electrophoresis, the gels were fixed with methanol/acetic acid/trichloroacetic acid/water (3:1:1:5, by vol.), impregnated

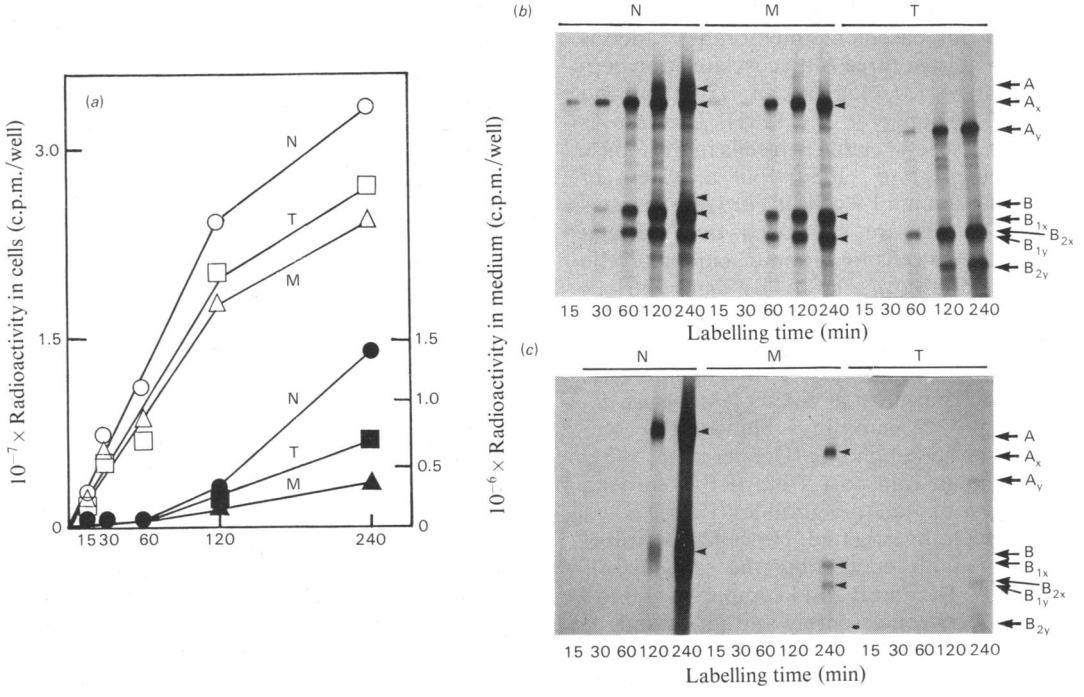


Fig. 1. *Kinetics of synthesis and processing of laminin subunits*

F9 cells were plated in a 96-well Multitest plate at a density of 10^4 cells/cm^2 and cultured for 6 days in the presence of 0.1μM-retinoic acid and 1mM-dibutyryl cyclic AMP. Resultant parietal endoderm-like F9 cells (3.0×10^5 cells/well) were pretreated for 2h with no addition (N), 1μM-monensin (M) or tunicamycin (1μg/ml) (T), and were labelled with [^{35}S]methionine for the indicated period in the presence or absence of antibiotics at the same concentration as during pretreatment. The labelled medium was collected and cells were lysed with a detergent solution containing iodoacetamide. (*a*) Trichloroacetic acid-insoluble radioactivity in media (●) and cell lysates (○) was determined. The immunoprecipitated laminin from labelled cell lysates (*b*) and media (*c*) corresponding to 7.5×10^4 cells were extracted by heating in the SDS sample buffer containing 2-mercaptoethanol, and analysed by reducing SDS/polyacrylamide-gel electrophoresis and fluorography as described in the Experimental section. Estimated M_r values for laminin subunits and their precursors were: A, M_r 370000; A_x, 350000; A_y, 320000; B, 230000; B_{1x}, 225000; B_{2x}, 215000; B_{1y}, 210000; B_{2y}, 190000.

with En^{3}Hance (New England Nuclear), dried, and exposed to X-ray films (Kodak XAR-5) at −80°C.

Results and discussion

In the present investigation, parietal endoderm-like F9 cells were labelled with [^{35}S]methionine in the absence or presence of monensin (1 μM) or tunicamycin (1 μg/ml) after 2h of pretreatment with these antibiotics. Assembly and glycosylation of laminin subunits were explored on the basis of their migration distance on SDS/polyacrylamide-gel electrophoresis under non-reducing and reducing conditions. As shown in Fig. 1(*a*), incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble fraction of cell lysates was linear up to 2h, but was not linear thereafter. However, even after 4h, 70% incorporation of the extrapolated value of initial linear parts was still observed in either the absence (N) or presence of monensin (M) or tunicamycin (T). Monensin and tunicamycin inhibited the incorporation into cell lysates by 30 and 20% respectively throughout the labelling period. Secretion of radioactive proteins into the medium was observed after a 2h lag period. On the basis of these results, two-dimensional electrophoresis for studying subunit assembly (Fig. 2) was performed on samples labelled for 4h.

Radiolabelled laminin subunits for SDS/polyacrylamide-gel electrophoresis were isolated with antiserum against whole laminin. The specificity of immunoprecipitation was checked by replacement of radiolabelled proteins with unlabelled laminin or by control precipitation with non-immunized serum. We confirmed that the immunoprecipitation of laminin was quantitative by comparing the fluorogram of SDS/polyacrylamide-gel electrophoresis of labelled medium before and after the immunoprecipitation (results not shown).

Figs. 1(*b*) and 1(*c*) present the migration distances of laminin subunits and their precursors on SDS/polyacrylamide-gel electrophoresis under reducing conditions. Mature subunits A and B (B_1 plus B_2; they cannot be separated well) were observed after 2h of labelling in the medium without antibiotics (Fig. 1*c*, lanes under 'N'). In the cell lysate (Fig. 1*b*, lanes under 'N'), however, a series of cytoplasmic precursors (A_x, B_{1x} and B_{2x}) was observed in addition to mature subunits.

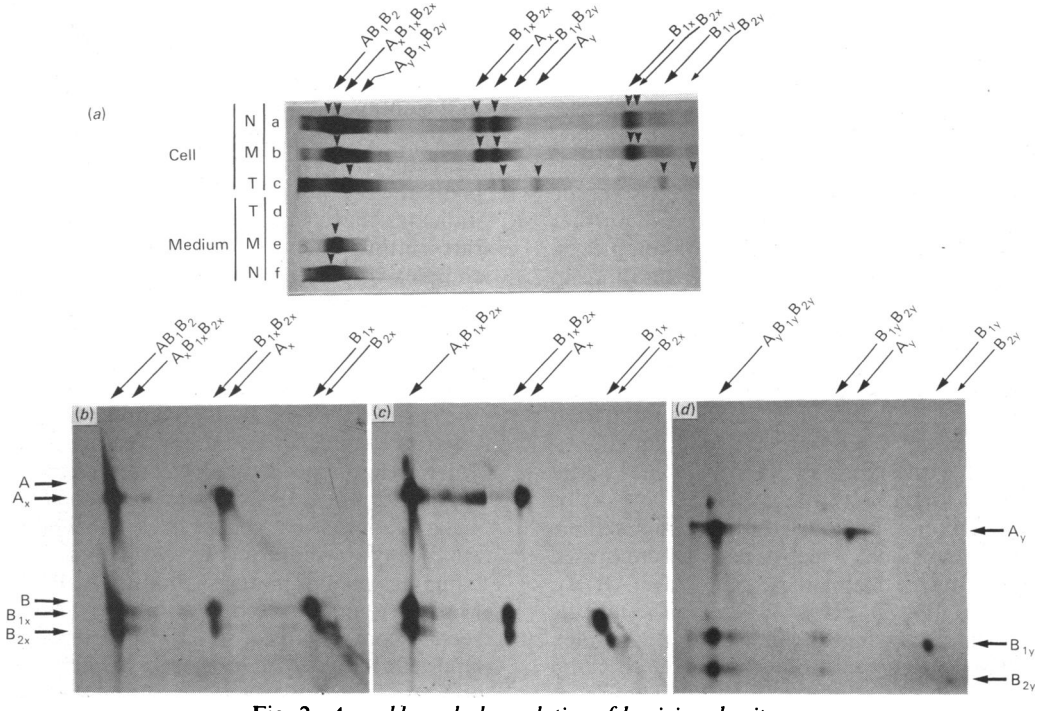


Fig. 2. *Assembly and glycosylation of laminin subunits*

Parietal endoderm-like F9 cells were pretreated with no addition (N), monensin (M) or tunicamycin (T) and labelled for 4h as described in Fig. 1. (*a*) Immunoprecipitated laminin from cell lysates (lanes a–c) and media (lanes d–f) corresponding to 7.5×10^4 cells were analysed by single-dimension electrophoresis under non-reducing conditions as described in the Experimental section. Immunoprecipitated laminin from cell lysates (7.5×10^4 cells) pretreated with no addition (*b*), monensin (*c*) or tunicamycin (*d*) were electrophoresed from left to right under non-reducing conditions and from top to bottom under reducing conditions.

Incorporation of radioactivity into these cytoplasmic precursors was linear up to 60 min and reached a steady state at the time when the secretion of mature subunits into medium was observed (2 h). As reported by Cooper *et al.* (1981), these cytoplasmic precursors were sensitive to endo-β-*N*-acetylglucosaminidase H (result not shown), indicating that they are bearing high-mannose type oligosaccharide chains. In the presence of monensin, on the other hand, only cytoplasmic precursors were observed in cell lysates (Fig. 1*b*, lanes under 'M'). Under this condition, these cytoplasmic precursors are secreted into medium without further processing (Fig. 1*c*, lanes under 'M'), although the amount was less than 10% of that of mature subunits from control cells. Tartakoff (1982) pointed out that monensin blocks the exit of secretory proteins from Golgi cisternae by perturbing their structures. As it was on IgM (Tartakoff & Vassalli, 1979) and fibronectin (Ledger *et al.*, 1983), processing of oligosaccharide side chains of laminin is probably inhibited by this effect of monensin. In the presence of tunicamycin, another series of precursors with lower M_r values (A_y, B_{1y}, and B_{2y}) was observed in cell lysates (Fig. 1*b*, lanes under 'T'). Considering the effect of tunicamycin (Takatsuki *et al.*, 1971), these may correspond to unglycosylated precursors. The result in Fig. 1(*c*) indicates that extremely small amounts of these unglycosylated precursors are also secreted into the medium.

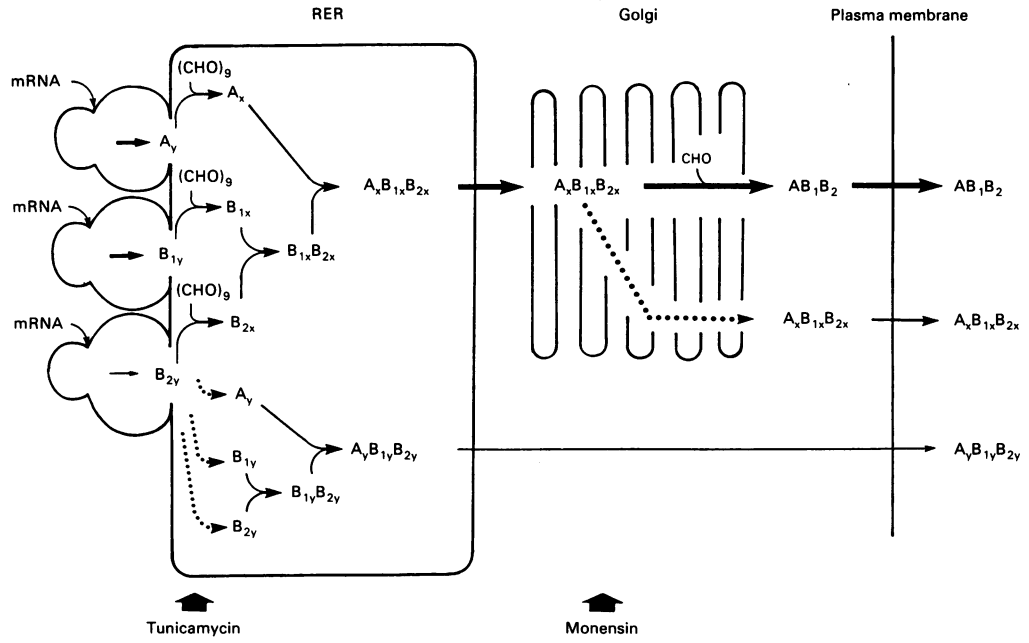
To study the assembly of subunits into complexes, radiolabelled laminin was analysed by non-reducing electrophoresis, preserving disulphide bondings between subunits (Fig. 2*a*). For further analysis of the subunit composition of complexes, bands separated under non-reducing conditions in the first dimension were further separated into subunits under reducing conditions in the second dimension (Figs. 2*b*–2*d*).

Analysis of cell lysates by non-reducing electrophoresis (Fig. 2*a*, lanes a–c) demonstrated that most of the radiolabelled laminin subunits migrated as bands with extremely high M_r values under all three conditions in the absence (lane a) or presence of monensin (lane b) or tunicamycin (lane c). Two-dimensional electrophoresis confirmed that the compositions of these bands are AB_1B_2, $A_xB_{1x}B_{2x}$ or $A_yB_{1y}B_{2y}$ (Fig. 2*b*–2*d*), although the spots of A and B (B_1 plus B_2) in the AB_1B_2 complex were not separated well from spots of A_x, B_{1x} and B_{2x} in the $A_xB_{1x}B_{2x}$ complex (Fig. 2*b*). These suggest that the assembly of subunits into ternary complexes (AB_1B_2, $A_xB_{1x}B_{2x}$ or $A_yB_{1y}B_{2y}$) is rapidly completed in some site in the cytoplasm. Considering the localization of protein disulphide-isomerase at the lumen side of RER (Ohba *et al.*, 1981), disulphide-bond formation between laminin subunits may be completed before they leave the RER. It is well established for many *N*-glycosylated proteins that transfer *en bloc* of high-mannose oligosaccharide side chains proceeds co-translationally (Hanover & Lennarz, 1981). Ternary complex formation in the presence of tunicamycin (Fig. 2*a*, lane c, and Fig. 2*d*) demonstrates that inhibition of this oligosaccharide transfer by tunicamycin does not affect the next step of disulphide-bond formation between laminin subunits. On the other hand, high-mannose oligosaccharide side chains seem to have a profound effect on the stability of polypeptides of laminin subunits. Amounts of radiolabelled laminin bands with lower M_r values than that of the ternary complex were very low in the cell lysate labelled in the presence of tunicamycin (Fig. 2*a*, lane c). As postulated for other *N*-glycosylated proteins (Olden *et al.*, 1978; Dulis *et al.*, 1982), high-mannose oligosaccharide chains seem to protect laminin polypeptides from proteolytic degradation.

Two-dimensional electrophoresis demonstrated the presence of $B_{1x}B_{2x}$ or $B_{1y}B_{2y}$ complexes in cell lysates labelled in the absence (Figs. 2*b* and 2*c*) or presence (Fig. 2*d*) of tunicamycin respectively. This suggests that the sequence of assembly of subunits is through $B_{1x}B_{2x}$ to $A_xB_{1x}B_{2x}$ and through $B_{1y}B_{2y}$ to $A_yB_{1y}B_{2y}$. Two-dimensional electrophoresis also demonstrated the presence of monomer pools of A_x, B_{1x} and B_{2x} in the cell lysate in the absence of tunicamycin (Fig. 2*b* and 2*c*) and pools of A_y, B_{1y}, and B_{2y} in the presence of tunicamycin (Fig. 2*d*). Among these monomer pools, the B_{2x} or B_{2y} pool was extremely small. This indicates that synthesis of B_2 polypeptides is the rate-limiting step for the assembly of laminin complex in parietal endoderm-like F9 cells. Cooper *et al.* (1981) reported somewhat different results for intracellular monomer pools. By their single-dimension electrophoresis, only pools of subunits A and B_2 were observed in P.Y.S. cells. We suspect that the $B_{1x}B_{2x}$ complex might not be separated from A_x, and the separation of B_{1x} and B_{2x} was not good in their electrophoresis.

An important observation in two-dimensional electrophoresis of cell lysates labelled in the absence of antibiotics (Fig. 2*b*) is that there is no monomer pool of mature subunits (A, B_1 and B_2). Mature subunits A and B (B_1 plus B_2) can only be seen as components of ternary complexes (Fig. 2*a*, lane a, and Fig. 2*b*). This clearly demonstrates that only ternary complexes of laminin go through intracellular traffic from RER to the site of terminal glycosylation, which is known to be localized in *trans*-cisternae of the Golgi apparatus (Tartakoff, 1982).

Despite perturbation of the Golgi cisternae by monensin, small amounts of laminin subunits



Scheme 1. *Sequence of post-translational assembly and glycosylation of laminin subunits*
The nomenclature for precursors of laminin subunits is as follows: A_x, B_{1x} and B_{2x}, precursors bearing high-mannose-type oligosaccharide side chains; A_y, B_{1y} and B_{2y}, unglycosylated precursors immediately after co-translational cleavage of signal sequences. $(CHO)_9$, core oligosaccharide side chain transferred *en bloc*; (CHO), monosaccharide. Dotted lines indicate inhibition site by tunicamycin or monensin.

could be secreted into the medium as seen in Fig. 1(*c*). A lesser amount of subunits could also be secreted even when glycosylation was inhibited by tunicamycin. Analysis of the medium by non-reducing electrophoresis demonstrated that only ternary complexes (AB_1B_2, $A_xB_{1x}B_{2x}$, or $A_yB_{1y}B_{2y}$) are secreted under all conditions (Fig. 2*a*, lanes d–f). In the medium labelled in the presence of tunicamycin (Fig. 2*a*, lane d), only a band of $A_yB_{1y}B_{2y}$ could be seen after prolonged exposure of the gel. But prolonged exposure did not show any laminin bands with M_r values lower than that of the ternary complexes in all media. These again indicate that only ternary complexes can pass the intracellular traffic from the RER to secretory granules.

Scheme 1 presents a summary diagram of our conclusions concerning post-translational assembly and glycosylation of laminim subunits. These include the notions that: (1) core oligosaccharide chains transferred *en bloc* are not necessary for subunit assembly; (2) assembly might proceed through B_1B_2 to AB_1B_2; (3) synthesis of B_2 polypeptides might be the rate-limiting step for assembly; and (4) only the AB_1B_2 complex undergoes oligosaccharide side-chain processing.

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