Analysis of the Assembly of Laminin and the Laminin–Entactin Complex with Laminin Chain Specific Monoclonal and Polyclonal Antibodies†

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ABSTRACT: Antibodies specific for the A, B1, and B2 chains of laminin have been obtained and characterized. Lam V, a rat × mouse monoclonal antibody, was obtained by immunizing Lewis rats with the extracellular matrix derived from the mouse endodermal line M1536-B3. The antibody was shown to recognize a conformation-sensitive epitope present on the A chain of laminin. The antibody exhibited high avidity for native laminin and uncomplexed newly synthesized laminin A chains. cDNA clones in the vector λ-gt11 containing sequences for the B1 and B2 chains of laminin were shown to synthesize β-galactosidase fusion proteins in the host cells induced with IPTG. The fusion protein F3 contained amino acid residues 822–1765 of the B1 chain of mouse laminin, and the fusion protein E4 contained 219 amino acids at the carboxyl terminus of the B2 chain of rat laminin. These two fusion proteins were used to obtain rabbit polyclonal antibodies which were characterized for their specificity and ability to immunoprecipitate laminin and the B chains of laminin. The chain-specific antibodies were used to analyze the assembly and processing of laminin in the mouse endodermal cell line M1536-B3. The results indicated that the covalent assembly of the A and B chains of laminin was initiated as early as 3 min after labeling cells. At this time point uncomplexed A chain of laminin could be observed even though there was an excess of B1 and B2 chains. As early as 4 min after labeling monomeric, dimeric, and oligomeric forms of the B chains of laminin were observed. Free B1 and B2 chains could also be detected after short labeling times. Sequential immunoprecipitation with the two B chain specific antibodies demonstrated that laminin complexes containing the A chain complexed to B1 alone, B2 alone, or both together were present in M1536-B3 cells. These observations support the notion that the assembly of laminin occurs initially by random association of the individual chains. The transit of laminin to a protease-sensitive extracellular compartment required approximately 60 min. The assembly of the laminin chains appeared to be independent of N-glycosylation since the assembly was almost as rapid in the presence of tunicamycin as in its absence. However, the turnover or exocytosis of the unglycosylated laminin was markedly retarded. The formation of the complex between laminin and entactin was examined; this complex could be observed as early as 5 min after labeling. The complex was exocytosed in approximately 1 h. These results suggested that the formation of the laminin–entactin complex was necessary for the transport of entactin to the extracellular compartment.

Laminin is a major glycoprotein component of basement membranes (Martin & Timpl, 1987). It consists of three polypeptide chains (A, B1, B2) linked by disulfide bonds (Hogan et al., 1982a). The glycosylated polypeptide chains migrate on SDS–polyacrylamide gels under reducing conditions with apparent molecular mass of 400 kDa (A) and 210–230 kDa (B2, B1). The laminin molecule has the characteristic and unique appearance of a cross when rotary-shadowed samples are viewed in the electron microscope (Engel et al., 1981). A model for the molecule has been proposed in which the carboxyl ends of the B1 and B2 chains and probably the A chain form a coiled-coil structure to give rise to the elongated segment of the long arm, while each of the short arms is contributed by one of the amino-terminus ends of the three chains (Barlow et al., 1985; Paulsson et al., 1985). The complete amino acid sequences of the B1 (Sasaki et al., 1987) and B2 chains have been determined (Sasaki & Yamada, 1987; Durkin et al., 1988). The distribution of the cysteine residues indicates that the two chains are held together covalently by three disulfide bonds, one at the carboxyl end and two near the intersection of the arms of the cross. Although the two B chains are derived from separate genes (Elliot et al., 1985), remarkably similar folding patterns are predicted from their amino acid sequences (Sasaki & Yamada, 1987).

Laminin interacts with itself (Yurchenko et al., 1985) and with other macromolecular components of the basement membrane including type IV collagen (Charonis et al., 1985, 1986), heparan sulfate proteoglycans (Woodley et al., 1983; Laurie et al., 1986), and entactin (Carlin et al., 1983; Paulsson et al., 1987). The interactions between these and other components yet to be identified contribute to the supramolecular organization of the basement membrane.

Laminin has been shown to enhance cell attachment (Terranova et al., 1980; Johansson et al., 1981; Carlsson et al., 1981), cell motility (McCarthy et al., 1983; McCarthy & Furcht, 1984; Graf et al., 1987), neurite extension (Baron-van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983; Edgar et al., 1984; Liesi et al., 1984), and neuronal survival (Thoenen & Edgar, 1985). The interaction of laminin and cell surface receptors is also believed to be involved in tumor metastasis (Liotta, 1984). The macromolecular and cell binding activities of laminin have been assigned to different domains of the molecule.

Laminin is synthesized by a variety of epithelial cells (Martin & Timpl, 1987). The mechanism of its biosynthesis,

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however, has not been studied in great detail. The biosynthetic pathway includes N-glycosylation of the three chains, formation of both intra- and interchain disulfide bonds, assembly of the two- or three-chain coiled-coil structure at the carboxyl ends, sorting of the chains to give the correct stoichiometry of A, B1, and B2, intracellular transport and processing, and finally exocytosis and incorporation into the extracellular matrix. Cooper et al. (1981) estimated that the laminin molecule is assembled within 10 min after mouse parietal endoderm cells are labeled. These results were confirmed by Peters et al. (1985), using human choriocarcinoma cells as a model system. The latter investigators suggested that a dimer of B chains was an obligatory intermediate and the A chain was the rate-limiting component in the assembly. Glycosylation of the chains was apparently not a factor in this process (Peters et al., 1985; Howe, 1984; Morita et al., 1985). In all of these experiments, the antibodies that were employed for immunoprecipitation of antigens were not chain specific. It was therefore difficult to follow the fates of the chains separately. The problem was compounded since the B chains existed in several intermediate forms that were not readily distinguishable by polyacrylamide gel electrophoresis.

Entactin is a sulfated glycoprotein of $M_r \sim 150\,000$ (Carlin et al., 1981; Hogan et al., 1982b; Timpl et al., 1983; Paulsson et al., 1986) which forms a very stable complex with laminin (Carlin et al., 1983; Paulsson et al., 1987). It is a widely distributed component of basement membranes (Bender et al., 1981; Martinez-Hernandez & Chung, 1984; Timpl et al., 1983). Entactin itself promotes attachment of epidermal cells (Alstadt et al., 1987) and appears in early mouse embryogenesis (Wu et al., 1983; Dziadek & Timpl, 1985). It has been shown that entactin modifies the cell attachment properties of laminin (Aumailley et al., 1987). The details of the formation of the laminin–entactin complex and its physiological consequences have not been explored adequately.

The production and characterization of chain-specific antibodies for laminin and their use in examining the early and late events in the biosynthesis of laminin and the laminin–entactin complex are the subject of this paper.

Materials and Methods

Cells. The mouse parietal endoderm cell M1536-B3 was isolated from differentiated embryonal carcinoma PCC4-F cells as previously described (Chung et al., 1977a). These cells which produce large quantities of laminin and entactin were grown in Dulbecco's modified Eagle medium supplemented with 10% bovine fetal calf serum (Gibco, Grand Island, NY) at 37 °C in a humidified 5–7% CO_2 atmosphere. In the majority of experiments described, the cells were first grown as monolayers and then after trypsinization transferred to nonadherent bacterial-type Petri dishes to facilitate harvesting and labeling with radioactive precursor amino acids.

Monoclonal Antibodies. The hybridoma which produced the A chain specific monoclonal antibody Lam V was obtained by immunizing Lewis rats with the extracellular matrix isolated from M1536-B3 cell cultures as described by Chung et al. (1977b). The procedure for the immunization of animals and the production of hybridomas were essentially those used previously to generate hybridomas that produced B chain specific antibodies (Chung et al., 1983). The monoclonal antibodies were screened by indirect immunofluorescent staining of M1536-B3 cells (Chung et al., 1979), enzyme-linked immunosorbent assays using as target microtiter wells coated with solubilized extracellular matrix (Chung et al., 1983), effectiveness in precipitating metabolically labeled laminin, and reactivity with 6 M urea extracted laminin adsorbed on nitrocellulose paper (Millipore Corp., Bedford, MA) as determined by an enzyme-linked assay (Hawkes et al., 1982) employing a rabbit anti-rat peroxidase-conjugated secondary antibody (Cooper Biomedical, Malvern, PA).

β-Galactosidase–Laminin Fusion Proteins. Two fusion proteins were obtained from cultures of induced lysogenized Y1089 *Escherichia coli* cells. The β-galactosidase–laminin B2 fusion protein E4 was produced by cells harboring the cDNA clone λ E4 that consisted of 3900 base pairs of rat sequences for the B2 chain of laminin. This cDNA clone has an open reading frame for 219 amino acids at the carboxyl terminus of the B2 chain; the remaining sequences were in the untranslated 3′-end of the laminin message. This clone was identified by screening a rat muscle λ gt11 cDNA library with antiserum containing anti-laminin antibodies (B. E. Carlin, and J.-P. Merlie, unpublished results) by the procedures of Young and Davis (1983). The insert was subcloned into Bluescript (Stratagene, San Diego, CA) and its 5′-end sequenced by the chain termination method of Sanger et al. (1977). The 5′-sequences were essentially identical with the sequence previously determined for the mouse laminin B2 cDNA clones (Barlow et al., 1984; Sasaki & Yamada, 1987; Durkin et al., 1988) and began at nucleotide 4349 at an internal *Eco*RI restriction site. The synthesis of the fusion protein was induced with IPTG as described by Young and Davis (1983). The bacterial cell extract was resolved on polyacrylamide gels in SDS according to the methods of Laemmli (1970). The proteins were transferred to Immobilon (Millipore, Bedford, MA) as described by Towbin et al. (1979), and the fusion protein was identified by staining with polyclonal anti-laminin antibodies by conventional enzyme-linked secondary antibody procedures (Chung et al., 1979). The position of the fusion protein relative to the bacterial proteins could readily be determined by comparison of the immunoblot with a parallel blot stained with Amido Black. In subsequent gels the fusion protein was visualized by treatment of the unstained gel with 1 M KCl. The protein band was excised from several runs, pulverized, and used to immunize New Zealand white rabbits by standard procedures (Carlin et al., 1981). The β-galactosidase–laminin B1 fusion protein, F3, was obtained from the cDNA clone λ F3. This clone was isolated from a cDNA library constructed with poly(A+) RNA from M1536-B3 cells (Durkin et al., 1986). The clone was identified by screening with serum containing anti-laminin antibodies. The sequence of the insert began at nucleotide 2823 of the B1 chain (Sasaki et al., 1987) and extended to the poly(A) tail of the message. The open reading frame included amino acids 822–1765 and began just within domain III of the B1 chain (Sasaki et al., 1987). This fusion protein was readily identified by its electrophoretic mobility and reactivity with anti-laminin polyclonal antibodies as described above. It was used to prepare anti-laminin B1 polyclonal antibodies in rabbits. The antibodies thus prepared were screened by their reaction with laminin in immunoblots, immunofluorescent staining of M1536-B3 cells, and their ability to precipitate metabolically labeled laminin.

Immunofluorescent Staining of M1536-B3 Cells. M1536-B3 cells were grown for 2 days on glass cover slips in 35-mm tissue culture dishes; at the end of that time the cell layers were rinsed three times with PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl), fixed with cold ethanol, and rinsed six times with cold PBS. The monoclonal or polyclonal antibody was applied to the cell layers at the appropriate dilution. Controls consisted of either antibody-free hybridoma culture medium or preimmune rabbit

serum. After incubation at room temperature for 1 h the antibody was removed, and the cells were rinsed six times with cold PBS; FITC-anti-rat IgG antibodies (Cooper Biomedical, Malvern, PA) were added, and the incubation was continued in the dark for another hour. The secondary antibody was removed, the cells were rinsed with six changes of PBS, and the cover slips were mounted cell side down on slides in mounting buffer (0.1 M glycine hydrochloride, pH 8.5, in 33% glycerol). The slides were then observed under fluorescent or phase-contrast microscopy.

Metabolic Labeling and Immunoprecipitation. M1536-B3 cells, seeded at a density of 2×10^6 cells per 100-mm dish, were grown in suspension culture for 24–48 h or as indicated in particular experiments. The cells from four dishes were harvested by centrifugation at 2000 rpm in a Sorvall GLC-2B centrifuge for 5 min, washed once with RPMI-1640 methionine-free medium (GIBCO, Grand Island, NY), and resuspended in 1 mL of the same medium. Tran^{35}S-label (>1000 Ci/mmol, containing a mixture of methionine and cysteine, ICN Biochemicals, Irvine, CA) was added to the cell suspension as indicated in individual experiments; aliquots of the suspension were immediately transferred to 1.5-mL plastic microcentrifuge tubes. After a predetermined incubation period the cells were rapidly separated by centrifugation for 10 s in a microcentrifuge. Depending on the experiment, the cell pellet was either immediately disrupted with lysing buffer or washed once, by resuspension and recentrifugation in complete DME supplemented with 10% FCS. In the latter case the radiolabel was then chased from the resuspended cells in unlabeled medium. The lysing buffer consisted of 10 μg/mL 1-[N^{α}-(*trans*-epoxysuccinyl)-L-leucylamido]-4-guanidinobutane (Sigma Chemical Co., St. Louis, MO), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% NP-40 (v/v), 1 mM EDTA, and 0.02% (w/v) sodium azide in PBS. The cell pellet was lysed with 150 μL of lysing buffer and kept on ice for 30–60 min with occasional mixing. An aliquot of 10 μL was transferred into a 1.5-mL microcentrifuge tube, 20–200 μL of pretitrated antibody solution or preimmune rabbit serum was added, and the immune complex was allowed to form on ice for at least 1.5 h. The immune complex was precipitated with pretreated *Staphylococcus aureus* cells (BRL, Bethesda MD). The pretreatment of the *S. aureus* cells was as follows: 1 mL of a 10% suspension of cells was first washed with 5 mL of 1% Triton X-100 (v/v) in PBS by centrifugation. The cell pellet was resuspended in 1 mL of 10% (v/v) 2-mercaptoethanol–3% (w/v) SDS in PBS and heated to 95 °C for 30 min; the cells were collected by centrifugation for 10 min at 3000 rpm in a Beckman JB-6 centrifuge, washed once with 5 mL of 1% Triton X-100 in PBS, resuspended in 1 mL of 10% (v/v) 2-mercaptoethanol–3% (w/v) SDS in PBS, and heated as before. The bacterial cells were washed twice with 0.5% (v/v) Tween 20–0.05% (w/v) SDS in PBS and at least three times with PBS and then reconstituted to the original concentration with PBS. The monoclonal antibody–antigen complex was precipitated with *S. aureus* cells that were further treated with rabbit anti-rat IgG antibodies (Cooper Biomedical, Malvern, PA). A suspension of *S. aureus* cells was mixed with the anti-rat IgG antibody and the mixture allowed to stand for 1.5 h at 0 °C followed by washing twice with 0.5% (v/v) Tween 20–0.05% (w/v) SDS in PBS and three times with PBS. The washed bacteria were then reconstituted in PBS.

A pretitrated volume of the *S. aureus* cell suspension was added to the immune complex in the microcentrifuge tube, and the contents were thoroughly mixed. The immune complex, now tightly bound to the bacterial cells, and the supernatant solution were separated by centrifugation and separately collected. The cell pellet was washed four times with 0.5% (v/v) Tween 20–0.05% (w/v) SDS and then resuspended and boiled in electrophoresis sample buffer for analysis by PAGE.

Electrophoresis and Autoradiography. Samples for electrophoresis were dissolved by being boiled for 3 min in electrophoresis sample buffer [0.062 M Tris-HCl, pH 6.8; 2% (w/v) SDS; 5% (v/v) 2-mercaptoethanol; 10% (v/v) glycerol; 0.05% (w/v) bromophenol blue]. The discontinuous pH system of Laemmli (1970) was used with a 5–7.5% polyacrylamide gel slab using a Hoefer slab gel apparatus (Hoefer Scientific, San Francisco, CA) for routine separation of proteins. Electrophoretic transfer of the resolved proteins to Immobilon was carried out with a Trans-Blot apparatus (Bio-Rad, San Francisco, CA) in transfer buffer composed of 1.44% (w/v) glycine–0.3% (w/v) Tris base in 20% (v/v) methanol. The transfer was effected at a constant 36 V for 4 h or longer. Protein bands on the membrane were detected by staining for 5 min with 0.1% (w/v) Amido Black in 45% (v/v) methanol–10% (v/v) acetic acid in water; the background stain was removed with the above solvent.

After the gels were dried on 3MM Whatman filter paper with a Bio-Rad slab gel drier, radiolabeled bands were detected by exposure to Kodak SB 100 film. The film was developed after the appropriate exposure time in an automatic X-ray film processor. Gels for fluorography were treated as described by Bonner and Laskey (1970) with DMSO and impregnated with 2,5-diphenyloxazole prior to exposure at −70 °C.

Proteinase K Digestion of Extracellular Matrix. M1536-B3 cells grown in suspension were harvested by centrifugation and resuspended at a density of 3×10^6 cells/mL of 20 mM Tris-HCl (pH 8.0)–150 mM NaCl–5 mM EDTA. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the suspension at a final concentration of 100 μg/mL and the extracellular matrix digested for 30 min. At the end of this time the cells were collected by centrifugation and washed with PBS containing 2 mM PMSF.

RESULTS

Fusion Proteins. The fusion proteins F3(B1) and E4(B2) were identified in induced lysogens containing the B1 and B2 laminin sequences by comparing the proteins synthesized by IPTG-induced and -uninduced lysogens. The apparent molecular masses, 148 and 200 kDa, for E4(B2) and F3(B1) were consistent with those expected from the cDNA sequences. The identity of these proteins was further confirmed by immunostaining of protein blots with anti-laminin polyclonal antibodies as shown in Figure 1. Lanes 1–4 were stained with Amido Black and lanes 5–9 with rabbit polyclonal anti-laminin antibodies. As may be seen in the IPTG-induced cell extracts, both the E4(B2) (lanes 1 and 7) and the F3(B1) (lanes 3 and 5) antigens were recognized by the antibody. In this figure the corresponding uninduced cell extracts shown in lanes 6 and 8 did not contain any anti-laminin cross-reacting material. It may also be seen that the antibody did not react with *E. coli* β-galactosidase (lanes 2 and 9). The protein pattern for the uninduced F3(B1) lysogen is shown in lane 4.

Antibody Specificity. The specificities of the monoclonal antibody Lam V(A) and the polyclonal antibodies anti-E4(B2) and anti-F3(B1) were tested by several criteria. Each of the three antisera recognized M1536-B3 antigens, as determined by indirect immunofluorescent staining. In order to establish more definitively the antigens that were recognized by anti-F3(B1) and anti-E4(B2) antibodies in M1536-B3 cells, the preformed extracellular matrix was destroyed by protease

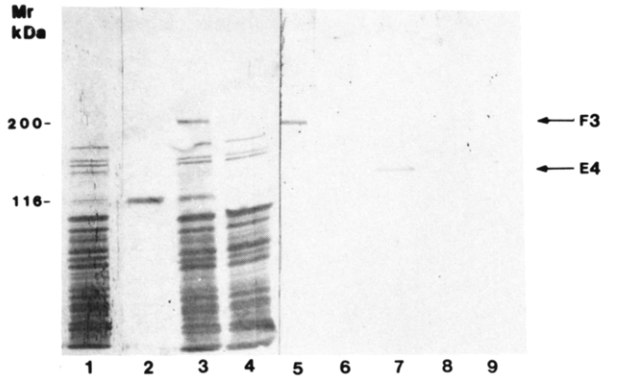

FIGURE 1: Immunoblot analysis of F3(B1) and E4(B2) fusion proteins. Proteins were transferred from a 7.5% polyacrylamide gel to Immobilon and stained with Amido Black, lanes 1–4, or with polyclonal anti-laminin antibodies (Chung et al., 1979), lanes 5–9. Lanes 1 and 7 contained cell extract of IPTG-induced λ-E4 lysogen; lanes 2 and 9, *E. coli* β-galactosidase; lanes 3 and 5, cell extracts of IPTG-induced λ-F3 lysogen; lanes 4 and 6, uninduced λ-F3 lysogen; lane 8, uninduced λ-E4 lysogen. Each lane contained 50 μg of protein except lanes 2 and 9 which contained 4 μg each.

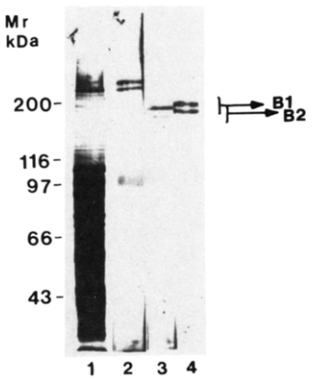

FIGURE 2: Immunostaining of laminin B1 and B2 subunits with anti-F3(B1) and E4(B2) antibodies. M1536-B3 cells were treated with 100 μg of proteinase K/mL of medium for 30 min at room temperature and then cultured in the presence of 5 μg of tunicamycin/mL of medium for 20 h. The cells were dissolved in sample buffer and the proteins separated on a 7.5% SDS–polyacrylamide gel. Lane 1 was excised, and the proteins were detected by staining with silver; the remaining lanes were transferred electrophoretically to Immobilon and stained with rabbit polyclonal anti-laminin A (lane 2), anti-E4(B2) (lane 3), and anti-F3(B1) (lane 4) antibodies.

digestion. The matrix-free cells were recultured in DME supplemented with 10% FCS and 5 μg/mL tunicamycin for 20 h. This was necessary since it was not possible to distinguish between the fully glycosylated B chains deposited in the matrix. The proteins in extracts from the tunicamycin-treated cells were resolved by PAGE, transferred to Immobilon, and stained with each antibody. As clearly shown in Figure 2, the two polyclonal antibodies recognized different protein bands: lane 2 was stained with a polyclonal A chain specific antibody, lane 3 with anti-E4(B2), and lane 4 with anti-F3(B1). In order to avoid any ambiguity, the transfer from a single gel lane was cut vertically into two pieces after alignment marks were made, and the two halves were stained separately with the two antibodies. The results show that anti-F3(B1) was specific for the B1 chain and anti-E4(B2) for the B2 chain. It is not obvious at this time why there were two bands for each of the laminin chains in the absence of N-glycosylation. Lane 1 contains the silver-stained bands from the tunicamycin-treated cells. The specificity of Lam V(A) was more difficult to establish since it did not react with the reduced denatured A chain on protein blots. It was found, however, that it preferentially immunoprecipitated metabolically labeled A chain as shown in Figure 3, lane 1. In this experiment M1536-B3

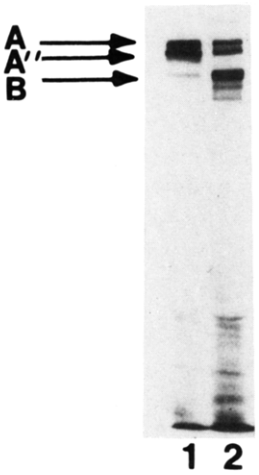

FIGURE 3: Specificity of Lam V(A) monoclonal antibody. M1536-B3 cells were labeled with Tran^{35}S-label (150 μCi/mL) for 4 min. Aliquots of the cell lysate (10 μL) were removed for immunoprecipitation with Lam V(A) (lane 1) and polyclonal anti F3(B1) antibodies (lane 2). The immunoprecipitates were analyzed by SDS–PAGE and autoradiography as described under Materials and Methods.

cells were labeled for 4 min before the immunoprecipitation with antibodies. In order to demonstrate that the faint band seen in lane 1 in the region of the B chain was probably due to the presence of a small amount of laminin complex, an aliquot of the cell extract was also treated with anti-F3(B1), the polyclonal B1-specific antibody, and as may be seen in lane 2, there was an abundance of B chains in the extract that was not recognized by Lam V(A). Lam V(A) will also recognize urea-extracted, but not SDS-extracted laminin as determined by dot blot immunoassays. These data suggest that it recognized a conformation-sensitive epitope. In both the immunoblot and immunoprecipitation experiments the preimmune serum did not react with the antigens.

Time Required for the Biosynthesis and Exocytosis of Laminin. It was possible to estimate the time required to chase radiolabeled laminin to the extracellular component. Cells were radiolabeled for 11 min and incubated in label-free medium for predetermined times. The extracellular matrix was removed with proteinase K, and the intracellular laminin was immunoprecipitated with either Lam V(A) or anti-F3(B1) antibodies. The data indicated that at 150 min of chase almost all the labeled laminin had entered the extracellular proteinase-sensitive pool. Several control experiments were included to ensure that under the conditions employed proteinase K effectively removed all the extracellular matrix proteins. For example, if the cells were lysed prior to protease treatment none of the matrix components was protected; furthermore, pretreatment of the enzyme with PMSF resulted in immunoprecipitation patterns that were identical with those obtained with untreated cells. Other experiments to be discussed later suggest that the time for exocytosis was somewhat shorter.

Time Required for Assembly of the Intact Laminin Molecule. The labeling and rapid cell harvesting procedures described under Materials and Methods combined with the A chain specific antibodies allowed us to examine the assembly process within 3 min of labeling. The results are presented in Figure 4. In Figure 4A, lane 1 shows that after 3 min of labeling the A chain and nascent fragments had incorporated radioactivity. At this early time there was a small amount of labeled B chain associated with the A chain. Treatment of the supernatant solution from this reaction time point with polyclonal anti-laminin B antibody (Chung et al., 1979), Figure 4B, lane 1, shows that there was a substantial amount of uncomplexed labeled B chain present. After labeling for 8 min

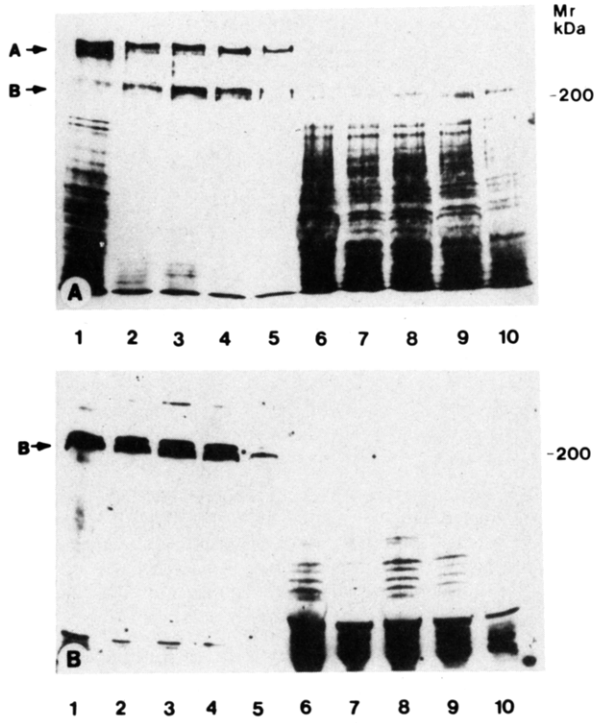

FIGURE 4: Time course of laminin biosynthesis. (Panel A) M1536-B3 cells were labeled with Tran^{35}S-label for 3 min (lane 1) or for 8 min and chased for 3 min (lane 2), 14 min (lane 3), 33 min (lane 4), and 62 min (lane 5). The labeled cell extracts were treated with Lam V(A) to precipitate both the free A chains and those complexed with B chains. The immunoprecipitates were solubilized in sample buffer, and aliquots were loaded on SDS–polyacrylamide gels to separate the labeled proteins, which were visualized by autoradiography. Lanes 6–10 contained aliquots of the supernatant solutions derived from the samples in lanes 1–5, respectively. In lanes 1 and 6, the amount of extract loaded was twice that in the other lanes. (Panel B) Aliquots of the supernatant solutions obtained after immunoprecipitation with Lam V(A) were treated with polyclonal anti-laminin B chain antibodies (Chung et al., 1979). The immunoprecipitates were dissolved and analyzed as described for lanes 1–5, panel A. The lanes and time points in panel B correspond to those in panel A. Lanes 6–10 were loaded with aliquots from the supernatant solutions obtained after immunoprecipitation with the anti-laminin B antiserum.

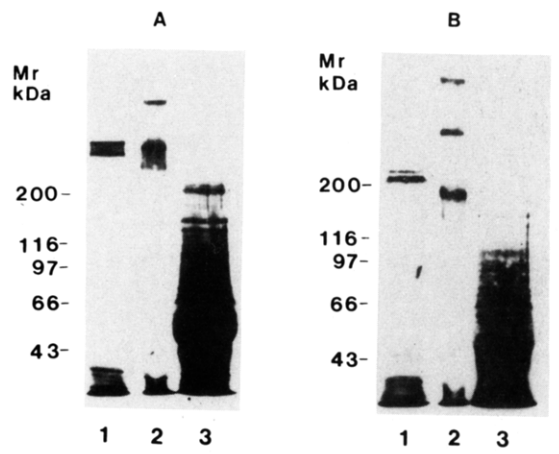

FIGURE 5: (Panel A) Covalent association of the A and B chains of laminin. M1536-B3 cells were labeled with Tran^{35}S-label for 4 min, and the cell lysate was treated with Lam V(A). Half of the immunoprecipitate was dissolved in sample buffer containing 2-mercaptoethanol (lane 1) and the other half in sample buffer from which the reducing agent was omitted (lane 2). The samples were analyzed in the usual manner on SDS–polyacrylamide gels followed by autoradiography. Lane 3 was loaded with the supernatant solution from the immunoprecipitation shown in lane 1. (Panel B) Covalent association of the B chains of laminin. The supernatant solution obtained after treatment of the sample shown in panel A, lane 2, was treated with polyclonal rabbit anti-laminin B chain antibodies (Chung et al., 1979). The immunoprecipitate was divided, and one portion was treated with 2% SDS–5% 2-mercaptoethanol (lane 1) and the other with 2% SDS alone (lane 2). The autoradiograms of the labeled bands are displayed. Lane 3 contains the supernatant solution after antibody treatment.

and 3 min of chase, Figure 4A, lane 2, more B chains became associated with the A chain. At all of the time points there was an excess of labeled B chains present as shown in Figure 4B, lanes 2–5. At 62 min of chase, lane 5 of panels A and B of Figure 4, about 40% of the radioactivity had disappeared from the complex. The chase times for lanes 2–5 were 3, 14, 33, and 62 min, respectively. Lanes 6–10 in Figure 4A are the supernatant solutions from lanes 1–5 after immunoprecipitation with Lam V(A). Lanes 6–10, Figure 4B, are the supernatant solutions from lanes 1–5 after immunoprecipitation with the polyclonal anti-laminin B antibodies (Chung et al., 1979). It may be concluded that the A chain became associated with the B chains within 3 min. This association could be noncovalent in nature since the immunoprecipitates were run in reducing gels. In order to determine if covalent association had occurred at this early time, the reaction product was precipitated with Lam V(A) after 4 min of labeling and run under both reducing and nonreducing conditions. It was observed that under nonreducing conditions a high molecular weight complex, Figure 5A, lane 2, typical of intact laminin was present; this high molecular weight species was absent upon reduction, Figure 5A, lane 1.

Multimeric Forms of Laminin B Chains. The presence of multimeric forms of laminin B chains was determined by labeling cells for 4 min, removing the laminin AB complex with Lam V, and treating the resulting supernatant solution with polyclonal anti-laminin antibodies. The immunoprecipitate was solubilized both with reducing and nonreducing sample buffer and analyzed by PAGE. As seen in Figure 5B, lane 1, the reduced sample yielded bands in the monomer B chain range, whereas the sample that was not reduced, lane 2, gave three bands that were interpreted to represent monomers, dimers, and oligomers. The unreduced monomeric form migrated somewhat faster than the reduced form, probably because it was more tightly folded. It appeared that the formation of B polymers was a very early event in laminin biosynthesis. The formation of oligomeric structures was unlikely to be an artifact since the inclusion of 0.1 M iodoacetamide during the extraction procedure yielded similar results.

Assembly of Unglycosylated Laminin. The assembly of the A and B chains of laminin was examined in cells treated with tunicamycin. The labeled cells, after solubilization, were treated in succession, first with anti-F3(B1) antiserum and next with anti-E4(B2) antiserum. The two successive immunoprecipitaes were then analyzed by PAGE. The converse experiment was also done in which the order of treatment with the two antisera was reversed. As displayed in Figure 6, panel A, lane 1, anti-F3(B1) precipitated an unglycosyated laminin complex which contained all three subunits, thus demonstrating that glycosylation was not obligatory for complex formation. In this lane any free B1 subunit would also be present. The supernatant solution from this experiment when subsequently treated with anti-E4(B2) antiserum yielded a laminin complex that consisted exclusively of the A and B2 chains of laminin. These results and those of the complementary experiment shown in lanes 2 and 3 demonstrate the existence of three distinct forms of laminin, AB1B2, AB1, and AB2. The unreduced immunoprecipitates from the second immunoprecipitation with anti-E4(B2) and anti-F3(B1) are shown in panel

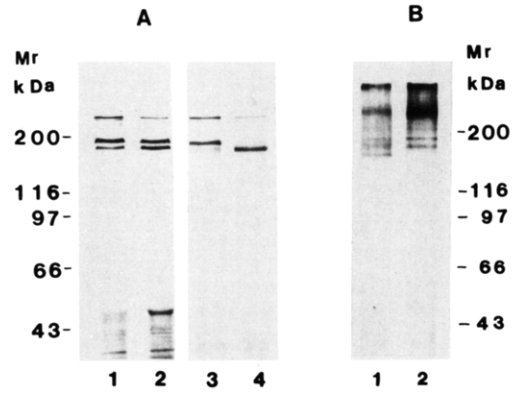

FIGURE 6: Assembly of unglycosylated A and B chains of laminin. M1536B3 cells (2×10^6) were grown overnight and then treated with tunicamycin for 3 h at a concentration of 5 μg/mL. The cells were washed once with RPMI-1640 without methionine but containing tunicamycin and resuspended in 1 mL of the same medium. After 10 min Tran^{35}S-label (120 μCi) was added to the medium, and the cells were incubated for 20 min. The cells were lysed as described under Materials and Methods. (Panel A) Aliquots of the lysate were immunoprecipitated with anti-F3(B1) antiserum (lane 1) or anti-E4(B2) antiserum (lane 2). The supernatant solutions from these reactions were next treated with anti-E4(B2) antiserum (lane 4) and anti-F3(B1) antiserum (lane 3), respectively. The immunoprecipitates were reduced before analysis by PAGE. (Panel B) The unreduced immunoprecipitates corresponding to lanes 4 and 3 in panel A were run in lanes 1 and 2, respectively.

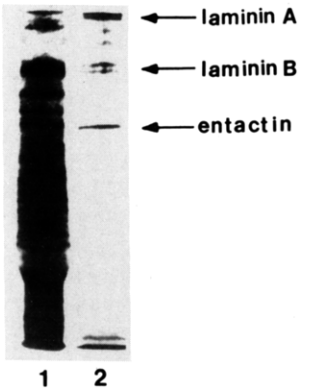

FIGURE 7: Formation of the laminin–entactin complex. M1536-B3 cells were labeled with Tran^{35}S-label for 5 min, and the cell extract was treated with rabbit polyclonal anti-entactin antiserum (Carlin et al., 1981). The immunoprecipitate (lane 2) and the supernatant solution (lane 1) were analyzed as described under Materials and Methods.

B, lanes 1 and 2, respectively. The high molecular weight species at the top of the gel support the conclusion that the A and B chains were covalently linked. Furthermore, the presence of free A and B species suggests that noncovalent assemblies were also present. Although the labeling time in these experiments was 20 min, in other experiments, using anti-F3(B1) or anti-E4(B2) antiserum, the laminin complex could be detected as early as 5 min after labeling. In addition, in the presence of tunicamycin the exocytosis of laminin was retarded as demonstrated by the retention of most of the laminin in the cells after 90 min of chase in unlabeled medium.

Laminin–Entactin Complex. The formation of the laminin–entactin complex was followed in the same manner as the formation of laminin from its individual chains. Entactin was synthesized in smaller amounts than laminin, however, as shown in Figure 7; immunoprecipitation of entactin with anti-entactin antiserum resulted in coprecipitation of laminin as early as 5 min after labeling, lane 2. Lane 1 displays the supernatant solution from the immunoprecipitation. The labeled entactin was chased into the protease-sensitive pool within 1 h.

Discussion

The assembly of the three chains of laminin and the formation of the laminin–entactin complex are important steps in the biosynthesis of basement membranes in general. To study these events in greater detail, we have developed an A chain specific monoclonal antibody, Lam V, and polyclonal antibodies to the B1 and B2 chains of laminin. The use of M1536-B3 cells and chain-specific antibodies has allowed us to gain new information on the details of the assembly and processing of laminin. The main conclusions that can be drawn from pulse–chase experiments combined with immunoprecipitation of the different biosynthetic intermediates and products with the specific antibodies are (a) the assembly and covalent linkage of the A and B chains were initiated as early as 3–4 min after their synthesis, (b) the laminin complex was transported to a protease-sensitive extracellular pool between 60 and 90 min after synthesis, (c) glycosylation was not necessary for the covalent assembly of the three chains of laminin, (d) the assembly of the chains appears to be random and did not require the obligatory formation of a B1B2 heterodimer or a specific AB dimer, and (e) the formation of the laminin–entactin complex occurred intracellularly.

A model for the assembly of the laminin subunits has previously been proposed (Peters et al., 1985). This model suggests that a B1B2 dimer is an obligatory intermediate in the assembly process. Our data do not support this model. Sequential immunoprecipitation of labeled laminin intermediates with B1 and B2 chain specific antibodies has clearly shown the presence of AB1, AB2, and AB1B2 laminin complexes. The assembly process does not appear to follow any obligatory order of association of the subunits. Our data instead suggest that the three chains of laminin are synthesized independently and initially assemble in a random manner. This is supported by the observation that unassociated A, B1, and B2 chains as well as B chain oligomers can be detected early in the synthetic pathway. These observations raise the possibility that the subunit composition of laminin in the extracellular matrix could be dependent on the prevailing concentrations of the individual subunits in the cisternae of the endoplasmic reticulum. These in turn would depend on the rate of expression of each gene and the rates at which their corresponding messages are translated. Since it is now known that each laminin subunit has its own characteristic functional properties, the ultimate behavior of the basement membrane will be dependent on the stoichiometry of the laminin subunits.

The rapid association and covalent linkage of the A and B chains suggest that the assembly occurs in the cisternae of the endoplasmic reticulum. At 3 min, the earliest time point in our labeling experiments, both complete and incomplete A chains were immunoprecipitated, and within 6 min most of these had been chased into the laminin complex. This time frame was inconsistent with the synthesis of the complete chains and transport to the Golgi complex for assembly. Addition of carbonyl cyanide (*m*-chlorophenyl)hydrazone, which inhibited endoplasmic reticulum–Golgi traffic partially blocked protein synthesis but did not prevent the assembly of laminin (unpublished results). The rapid assembly was confirmed by immunoprecipitation with anti-B1 and anti-B2 antibodies and in tunicamycin-treated cells. The subsequent processing of the complex was much slower since even after 60 min of chase in unlabeled medium a substantial fraction of the laminin remained in the protease-insensitive intracellular compartment. The assembly of the laminin chains in the

presence of tunicamycin was apparently unimpaired. However, in these cells the half-life of the labeled molecules was extended. This could be due to secondary effects of tunicamycin on the exocytosis or turnover of laminin or the requirement for glycosylation in transport. The results here were in agreement with those of Howe (1984).

The incorporation of radiolabel into entactin at the short time intervals used in the pulse–chase experiments rendered it more difficult to follow the kinetics of formation of the laminin–entactin complex. Hovever, those experiments in which anti-entactin antiserum was used as the immunoprecipitating reagent, with high levels of radiolabeling, indicated that the laminin–entactin complex was present after 5 min of labeling. The transit of entactin to the extracellular protease-sensitve compartment required approximately 60 min, which was similar to that for laminin. The presence of an intracellular complex of laminin and entactin suggested that there may be alternate pathways for the exocytosis of laminin, one route as uncomplexed laminin and the other as an entactin–laminin complex. Although it was attractive to consider the obligatory association of laminin with entactin as a secretory signal, the apparent rate of synthesis of entactin was lower than that of laminin. It appeared rather that entactin can only be exocytosed as a complex with laminin. The early formation of the complex suggested that it was assembled in the cisternae of the endoplasmic reticulum. It has been suggested (Aumailley et al., 1987) that entactin may modify the cell-binding properties of laminin. This was based on examination of the laminin–entactin complex by electron microscopy and also by in vitro cell binding studies. The regulation of the formation of the laminin–entactin complex may therefore have important physiological implications and must be explored in greater detail.

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Structural Changes in Vesicle Membranes and Mixed Micelles of Various Lipid Compositions after Binding of Different Bile Salts†

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ABSTRACT: Binding equilibria of common bile salts (BS) and different mixtures of membrane lipids were correlated with BS-induced structural changes of large unilamellar vesicles, with transition of vesicles to mixed micelles (MM), and with successive transformations of MM. At very low BS concentrations, in the outer vesicle monolayer definite BS/lipid aggregates are formed, the size and BS binding strength of which depend on the BS and lipid species involved. At increasing BS concentrations, binding to the membranes is hampered, and above a critical BS content, membrane stress due to asymmetric BS binding leads to formation of transient membrane holes, as shown by inulin release from the vesicles. Independent of the BS and lipid species, membrane solubilization starts at a ratio $r = 0.3$ of bound BS/lipid. Increasing phosphatidylserine, phosphatidylethanolamine, and cholesterol contents stabilize the lecithin membrane against BS to different degrees and in different ways, whereas the destabilization by sphingomyelin is probably due to the enhancement of the membrane gel–liquid transition temperature. Conjugation of the BS with glycine or taurine has a modulating effect on membrane hole formation, rather than on lipid solubilization. Diphenylhexatriene fluorescence anisotropy indicates a BS-induced drop of the internal membrane order and its restoration during membrane solubilization. At higher concentrations ursodeoxycholate induces additional condensation, whereas the other BS cause internal disorder in the MM. Above ratios r of approximately 8:1, we found a release of BS from these MM and suggest a rodlike structure for them. The results were discussed with respect to BS/membrane interactions during lipid excretion from the liver cell.

Bile salts play an important role both in the absorption of dietary lipids from the intestine and in the excretion of cholesterol from the liver into the intestine. They are highly efficient in dissolving cholesterol with the help of lecithin to form mixed micelles, the primary species of lipid aggregates in bile. Ruetz et al. (1987) recently demonstrated that bile salt excretion from the liver cell at the canalicular surface domain is driven by a negative membrane potential and occurs via a carrier glycoprotein. Cholesterol and lecithin are probably excreted as vesicles. The mechanism and the site of solubilization of these vesicles, however, are unknown. After fusion of the vesicles with the canalicular part of the liver cell plasma membrane (cLPM),[1] bile salts may specifically extract lipids from distinct membrane areas or may solubilize vesicles after their budding into the canalicular space.

Moreover, bile salts possibly bind to membranes of cell organelles and, after excretion, especially to the outside of the cLPM and interact with several membrane lipids. As postulated for some cholestatic diseases, disarrangement of the physiologic bile salt composition or disturbance of membrane protecting mechanisms, therefore, can result in severe cellular dysfunctions.

For further elucidation of the mechanisms of membrane/bile

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[1] Abbreviations: LUV, large unilamellar vesicle; Chol, cholesterol; EYL, egg yolk lecithin; PE, phosphatidylethanolamine; EYPE, egg yolk phosphatidylethanolamine; PS, phosphatidylserine; BPS, bovine brain phosphatidylserine; Sph, sphingomyelin; BSph, bovine brain sphingomyelin; EYSph, egg yolk sphingomyelin; PSph, porcine erythrocyte sphingomyelin; [^{14}C]DPPC, [^{14}C]dipalmitoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; OG, *n*-octyl β-D-glucopyranoside; BS, bile salt; cholate, 3α,7α,12α-trihydroxy-5β-cholanic acid; GC, glycocholate; TC, taurocholate; DC, deoxycholate (3α,12α-dihydroxy-5β-cholanic acid); GDC, glycodeoxycholate; TDC, taurodeoxycholate; CDC, chenodeoxycholate (3α,7α-dihydroxy-5β-cholanic acid); GCDC, glycochenodeoxycholate; TCDC, taurochenodeoxycholate; UDC, ursodeoxycholate (3α,7β-dihydroxy-5β-cholanic acid); GUDC, glycoursodeoxycholate; TUDC, tauroursodeoxycholate; LC, lithocholate (3α-hydroxy-5β-cholanic acid); GLC, glycolithocholate; TLC, taurolithocholate; cLPM, canalicular part of the liver cell plasma membrane; ^{13}C NMR, carbon-13 nuclear magnetic resonance; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance.

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