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Laminin biosynthesis in the extracellular matrix-producing cell line PFHR9 studied with monoclonal and polyclonal antibodies

Jean-Claude S. LISSITZKY,*‡ Colette CHARPIN,† Christophe BIGNON,* Madeleine BOUZON,* Francis KOPP,* Pierre DELORI* and Pierre-Marie MARTIN*

*Laboratoire de Cancérologie Expérimentale, UA CNRS 1175, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13326 Marseille Cedex 15, France and †Laboratoire de Neuroanatomopathologie, Faculté de Médecine, Boulevard Jean Moulin, 13005 Marseille Cedex 5, France

The biosynthesis of the basement-membrane glycoprotein laminin in the mouse teratocarcinoma cell line PFHR9 was studied by immunoelectron microscopy and pulse–chase experiments using monoclonal and polyclonal antibodies. By immunoelectron microscopy, most of the protein was found to be aggregated on the outer cell surface. Cytoplasmic stainings were rare and were located next to the intracellular side of the plasma membrane. Sequential immunoprecipitations of cell extracts with a monoclonal antibody (4C12) sensitive to the laminin native conformation and with a polyclonal antibody enables laminin, the B_1 subunit and a 410 kDa molecule to be distinguished. Most of the laminin is of the $A(B_1B_2)$ type, and the 410 kDa molecule appears to be a B_1B_2 heterodimer. The assembly of laminin from subunits is completed in less than 1 h, and B chains are incorporated via the formation of the B heterodimers. The B_2 and A chains are not found as free forms, so their levels appear to be the rate-limiting factors for the assembly of the dimers and laminin respectively. The formation of an uncross-linked $A(B_1B_2)$ complex as a short-lived intermediate in the biosynthetic process is possible. Together with immunoelectron microscopy, the present study suggests that the protein is rapidly exported after assembly to accumulate on the outer side of the cell membrane. The biosynthesis of laminin in the PFHR9 cell line appears to be similar to that in other matrix-producing cell lines.

Laminin (Timpl *et al.*, 1979) is a 900 kDa glycoprotein produced by several cell types, including endothelial cells (Gospodarowicz *et al.*, 1981), endodermal cells (Sakashita & Ruoslahti, 1980) and Swann cells in the central nervous system (Palm & Furcht, 1983). The protein is found adsorbed on the cell surface (Wicha & Huard, 1983; Charpin *et al.*, 1985) or deposited in the basement membrane (Foidart *et al.*, 1980), where it is in association with other basement-membrane components as a part of supramolecular complexes (Yurchenco *et al.*, 1986).

Laminin plays an important role in several aspects of cell biology. It has been found to modulate cell differentiation (Manthorpe *et al.*, 1983), cell shaping (Cody & Wicha, 1986) and also cell movement, because it appears to be an important cell substrate-adhesion protein (Terranova *et al.*, 1980; Hand *et al.*, 1985). The protein has been implicated in biological situations involving phenotype fluctuations. As a matter of fact, variations in the expression of the protein have been observed in embryogenesis (Liesi, 1985), organogenesis (Ekblom *et al.*, 1980), post-traumatic healing (Gulati *et al.*, 1983) and cancer (Albrechtsen *et al.*, 1981; Vlodavsky & Gospodarowicz, 1981). Therefore, the evaluation of laminin expression by cells would be an important step in understanding the mechanisms controlling these phenomena.

Laminin results from the disulphide cross-linking of several subunits [A (440 kDa), B_1 (225 kDa) and B_2 (205 kDa) (Cooper *et al.*, 1981)], each of which results from the activation of its own gene (Wang & Gudas, 1983; Barlow *et al.*, 1984; Durkin *et al.*, 1986). The manner in which the subunits assemble to form the molecule, and the number of subunits involved, is still a matter of controversy. Analysis of laminin biosynthesis in the mouse teratocarcinoma F9 (Morita *et al.*, 1985) and in the human choriocarcinoma JAR (Peters *et al.*, 1985) indicates that the AB_1B_2 structure prevails. However, the finding of a differential reaction of monoclonal antibodies with the basement membranes of mouse embryonic and adult tissues (Wan *et al.*, 1984), and the incomplete blockage by anti-laminin polyclonal antibodies of the laminin-related neurotropic activity contained in various conditioned media (Lander *et al.*, 1985), have raised the possibility that there are laminin isoforms differing in their structure and function. Furthermore, the immunodetection of laminin within human and mouse tissues shows that the protein is located most often in the extracellular spaces and less frequently intracellularly (Foidart *et al.*, 1980; Albrechtsen *et al.*, 1981; Charpin *et al.*, 1986).

In an effort to understand more of the details of laminin production, we have analysed the biosynthesis of this molecule in a mouse cell line of the endodermal type, PFHR9 (Chung *et al.*, 1977), by pulse–chase experiments and immunoelectron microscopy using monoclonal and polyclonal antibodies.

Abbreviations used: FCS, foetal-calf serum; PBS, phosphate-buffered saline (for composition, see the text); PAGE, polyacrylamide-gel electrophoresis, BSA, bovine serum albumin.

‡ To whom correspondence and reprint requests should be sent.

MATERIALS AND METHODS

Antibodies

The polyclonal antibody 1219 and the monoclonal antibody 4C12 (Immunotech, Marseille, France) were prepared, purified and their specificity tested as previously described (Charpin *et al.*, 1986). The polyclonal antibody was purified by affinity chromatography on protein A–Sepharose 4B (Pharmacia). The monoclonal antibody was purified by anion-exchange chromatography on DEAE-Trisacryl (IBF, Villeneuve-la-Garenne, France) or Mono Q (Pharmacia). Purified antibodies were coupled to CNBr-activated Sepharose 4B (Pharmacia) or Affigel 10 (Bio-Rad) according to the manufacturer's instructions, high-capacity immunosorbents thus being obtained.

Laminin

Laminin was extracted from the mouse EHS sarcoma and purified by anion-exchange chromatography (Timpl *et al.*, 1979). Laminin was purified from the PFHR9-cell-line conditioned medium by affinity chromatography on a polyclonal anti-laminin immunoglobulin–Sepharose 4B column. The adsorbed material was eluted with 0.1 M-NH_4HCO_3/0.25% SDS. A portion of the eluted material was further purified by gel filtration on a Superose 6 column (Pharmacia), also eluted with 0.1 M-NH_4HCO_3/0.25% SDS.

Cell labelling

The laminin-secreting cell line PFHR9 was maintained as described by Chung *et al.* (1977).

***(a)* Steady-state labelling.** This was performed by feeding the cells grown to early confluency in 100 mm-diameter Petri dishes (Falcon) for 8 h with 300 μCi of [^{35}S]methionine (> 1000 Ci/mmol; New England Nuclear) in methionine-free RPMI/1 μM-methionine/dialysed 10% (v/v) foetal-calf serum (FCS).

***(b)* Pulse labelling.** Cells grown to early confluency were washed with methionine-free RPMI/20 mM-Hepes and incubated in this medium (0.5 ml) 25 min before pulse–chase. The pulse was started by adding 100 μCi of [^{35}S]methionine (> 1000 Ci/mmol; New England Nuclear) to the preincubation medium. After 5 min of pulse, the medium was removed and the cells were 'chased' for various periods of time with RPMI/0.05 M-methionine/10% (v/v) FCS. All media used in pulse–chase experiments were heat-equilibrated and gas-saturated.

Immunoprecipitations

Cell lysates were obtained by lysing the cells in freshly prepared ice-cold 0.05 M-phosphate/0.15 M-NaCl, pH 7.4 (PBS) supplemented with methionine (0.2 M), Triton X-100 (1%, v/v) and proteinase inhibitors (2 mM-phenylmethanesulphonyl fluoride, 8 mM-*N*-ethylmaleimide and 3 mM-EDTA). The cell lysates were then briefly sonicated and centrifuged (5 min, 5000 *g*) to remove the insoluble material. The cell-conditioned medium was centrifuged (5 min, 1000 *g*) and the supernatant was supplemented with Triton X-100, methionine and proteinase inhibitors as described for the cell lysates. All operations were performed with the tubes on ice, and samples were processed immediately in order to avoid freeze–thawing.

Portions (0.1 ml) of thoroughly washed monoclonal or polyclonal immunosorbents were then added to the samples, and the mixture was placed overnight (+5 °C) on a roll-over shaker. For sequential immunoprecipitations, the supernatant of the monoclonal immunoprecipitates was divided into equal portions, which were immunoprecipitated again with the monoclonal or the polyclonal immunosorbents respectively (12 h, +5 °C). The immunosorbents were then washed several times in PBS/1% Triton X-100, and the immunoprecipitated material was eluted by boiling the sorbents in SDS/PAGE sample buffer.

Gel electrophoresis

SDS/PAGE. The immunoprecipitated material was analysed unreduced or reduced (0.01 M-dithiothreitol) and alkylated (0.04 M-iodoacetamide) by electrophoresis on 3–12%-(w/v)-polyacrylamide slab gels run in the discontinuous dissociating Laemmli buffer system (SDS/PAGE). A kit of high-M_r proteins (Sigma) was used as a source of migration standards. Gels were stained with Coomassie Blue, destained, impregnated with En^{3}Hance (New England Nuclear), dried, and exposed at −70 °C to preflashed Kodak X-Omat AR or Amersham MP films. When required, properly exposed autoradiograms were quantified by linear scanning with a Shimadzu CCSI-CS930 integrating densitometer.

Agarose electrophoresis. In some cases, the material to be analysed was electrophoresed in a vertical apparatus on 2% (w/v) agarose gels (agarose C; Pharmacia) with Tris (0.04 M)/sodium acetate (0.02 M)/acetic acid (0.033 M)/EDTA (0.002 M)/SDS (0.2%) as gel and running buffer. Blue-stained high-M_r proteins (BRL, Cergy Pontoise, France) were used as migration standards.

Measurement of the A- and B-subunit content of laminin and the 410 kDa species by two-step analysis

Laminin and the 410 kDa species were separated by ultracentrifugation or by first-dimension non-reducing SDS/agarose electrophoresis and were further analysed by second-dimension SDS/PAGE under reducing conditions.

Sucrose gradient–SDS/PAGE analysis. An extract of PFHR9 cells labelled with [^{35}S]methionine under steady-state conditions was layered on a gradient of sucrose [7–30%, (w/w) in Tris (0.05 M)/proteinase inhibitors, pH 8]. After centrifugation (SW60 rotor; 227000 *g*; 17 h; 4 °C) the gradient was fractionated, and fractions (0.1 ml) were immunoprecipitated with the monoclonal-antibody sorbent and analysed by reducing SDS/PAGE.

Analysis by two-dimensional SDS/PAGE. Laminin and the 410 kDa species in the material immunoprecipitated from the cell extracts during pulse–chase were separated by SDS/agarose electrophoresis (first dimension). EHS-sarcoma laminin unreduced and EHS-sarcoma laminin reduced and alkylated, were run in separate lanes as migration standards; these lanes were excised and stained with Amido Black to locate the migration positions of laminin and laminin A and B subunits. PFHR9-cell laminin, migrating as the fastest component in the ladder of electrophoretic bands displayed by EHS-sarcoma laminin on agarose electro-

phoresis, was well separated from the 410 kDa species. Portions of the lanes from the pulse–chase immunoprecipitates that corresponded to laminin and 410 kDa were excised and supplemented with SDS (2%), Bromophenol Blue and reducing agent [0.02 M-dithiothreitol or 5% (v/v)-β-mercaptoethanol], boiled, and analysed by SDS/PAGE.

Immunoblots

The material to be analysed by immunoblotting was electrophoresed on SDS/agarose gels and electrotransferred (10 V/cm 4 h) to nitrocellulose in 0.02 M-Tris/0.2 M-glycine(pH 8.3)/20% (v/v) methanol. After transfer, the nitrocellulose was blocked with BSA [2.5% (w/v) in PBS] (overnight, 4 °C), and the blotted material was tested for reactivity with the monoclonal or polyclonal antibodies (6 h, 20 °C). After several washes in PBS/Tween 20 (0.2%), the nitrocellulose was incubated with the relevant peroxidase-conjugated anti-(rat immunoglobulin) or anti-(rabbit immunoglobulin) second antibody (DAKO, Copenhagen, Denmark) [1:50 (v/v) in PBS/0.25% BSA]. After the washes, the blot was developed with 4-chloro-1-naphthol (0.5 mg/ml) in 25 mM-Tris/20% methanol, pH 7.5.

Immunoelectron microscopy

This was performed as previously described (Charpin *et al.*, 1986), with pre-embedding and avidin–biotin complex techniques (Vector Laboratories, Institut Pasteur, Paris, France).

RESULTS

Analysis of antibody specificity

The specificity of the anti-laminin antibodies, the rabbit polyclonal antibody 1219 and the monoclonal rat IgG1 4C12 previously described (Charpin *et al.*, 1986) was re-assessed by immunoblots of laminin and laminin fragments separated by electrophoresis on SDS/agarose gel. SDS/agarose electrophoresis was preferred to SDS/PAGE because high-M_r proteins, and laminin in particular, could be transferred much more efficiently to nitrocellulose from agarose than from acrylamide.

On agarose electrophoresis, laminin migrates as a set of bands; it is not known at present whether this behaviour results from protein–gel-matrix interactions or from a polymerization of the molecule involving links resistant to SDS denaturation. Notwithstanding, the immunoblot study confirms our previous data by showing that the polyclonal antibody reacts with laminin and its reduced and alkylated subunits (Fig. 1*a*). In contrast, 4C12, which does not recognize the A and B subunits, interacts with native laminin by the way of an epitope with a disulphide-dependent conformation (Fig. 1*b*).

Immunoprecipitation analysis

The SDS/PAGE analysis of the material immunoselected by the polyclonal antibody from the cell contents or the secretion medium of the PFHR9 cell line labelled under steady-state conditions yields results close to those obtained in similar experimental conditions in various

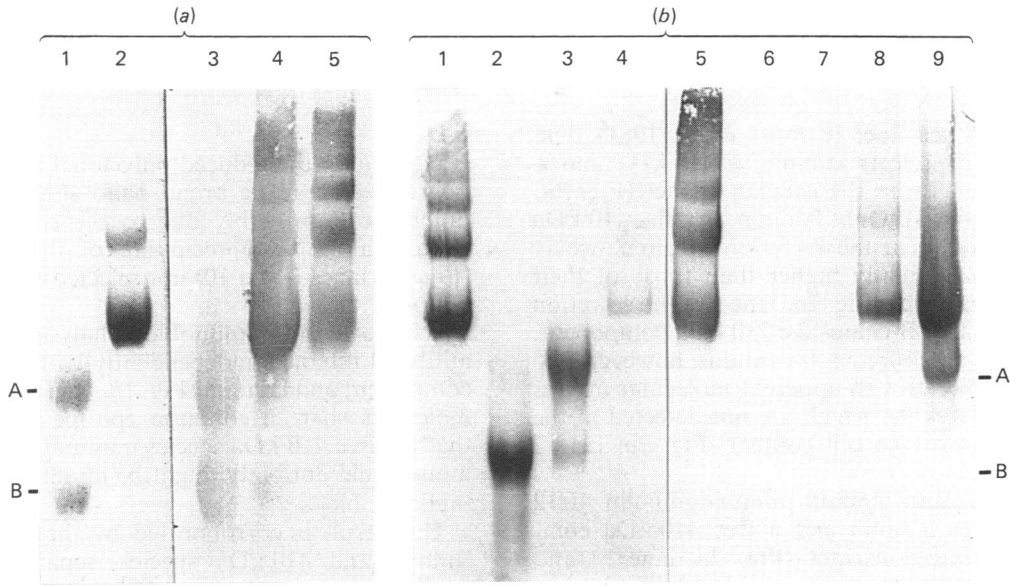

Fig. 1. Agarose-gel electrophoresis and immunoreactivity after electroblotting of EHS-sarcoma laminin and subunits with the rabbit polyclonal antibody 1219 and the rat monoclonal antibody 4C12

(*a*) EHS-sarcoma laminin was electrophoresed on 2.5% agarose gels: lanes 2, 4 and 5, unreduced; lanes 1 and 3, reduced and alkylated; lanes 1 and 2, Amido Black stain of the untransferred gel; lanes 3, 4 and 5, electrophoresed material electrotransferred to nitrocellulose and tested for reactivity with the polyclonal antibody. (*b*) lanes 1 and 5, EHS-sarcoma laminin; lanes 2 and 6, partially purified laminin B subunit; lanes 3 and 7, partially purified A subunit; lane 9, laminin-related material in the PFHR9 secretion medium extracted by affinity for the anti-laminin polyclonal antibody, then further purified by gel filtration on Superose 6 (lanes 4 and 8). Samples were electrophoresed on a 2.5%-agarose gel: lanes 1, 2, 3 and 4, Amido Black stain of the untransferred gel; lanes 5, 6, 7, 8 and 9, immunoblot with the monoclonal antibody of the material electrotransferred to nitrocellulose. A and B indicate the migration positions of the laminin A and B chains.

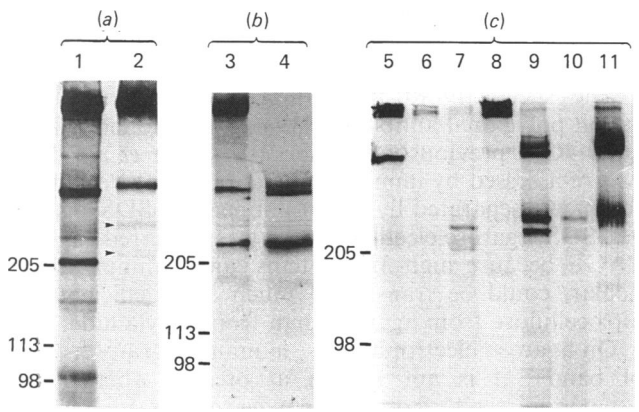

Fig. 2. SDS/3–12%-polyacrylamide-gradient gel analysis of the polyclonal and the monoclonal immunoprecipitates from the cell content or the secretion medium of the PFHR9 cell line labelled under steady-state conditions with [^{35}S]methionine

(*a*) Unreduced polyclonal immunoprecipitate of: lane 1, the cell contents; lane 2, the secretion medium (arrowheads point at the material in the medium not found in the cell lysate). (*b*) Polyclonal immunoprecipitate of the cell content: lane 3, unreduced; lane 4, reduced. (*c*) Monoclonal immunoprecipitate from the cell content: lane 5, unreduced; lane 9, reduced and alkylated; lanes 6, 7 and 10, material remaining in the supernatant after the immunoprecipitation of the cell content by the monoclonal antibody and selected by sequential immunoprecipitation using the monoclonal antibody (lane 6, unreduced) or the polyclonal antibody (lane 7, unreduced; lane 10, reduced and alkylated); lanes 8 and 11, direct immunoprecipitate of the secretion medium by the polyclonal antibody (lane 8, unreduced; lane 11, reduced and alkylated). Values on the left refer to the molecular masses (in kDa) of the protein used as migration standards.

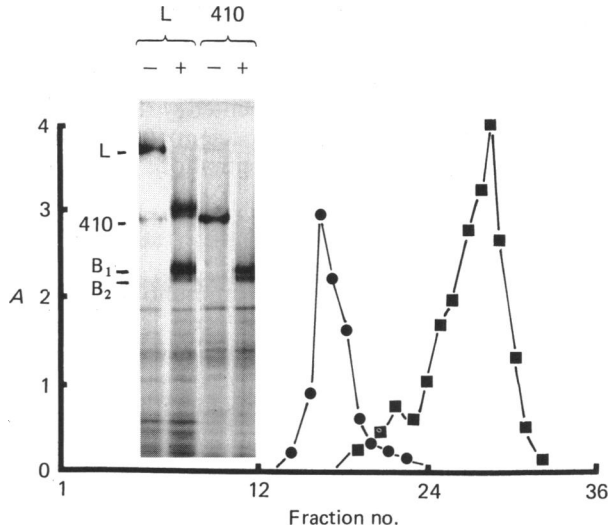

Fig. 3. Characterization of the PFHR9 410 kDa component selected by the monoclonal antibody

Main Figure: a [^{35}S]methionine-labelled cell extract was applied to a 7–30%-(w/v)-sucrose gradient, which was ultracentrifuged (227000 *g*, 17 h, 4 °C) on an SW60 rotor. The material immunoprecipitated from the gradient fractions (0.1 ml) was analysed by SDS/PAGE under non-reducing conditions. The fluorograms of the gels (not shown) were scanned by densitometry and the absorbances related to 410 kDa component (●) or laminin (■) were measured and are plotted as a function of their migration position in the gradient (fraction no.). The top of the gradient is fraction 1; the bottom is fraction 36. Inset electrophoretogram: the material in fractions 15 and 16 (410 kDa) and fractions 28 and 29 (laminin, L) was analysed by SDS/3–12% PAGE under non-reducing (−) and reducing conditions (+). L, 410, B_1 and B_2 in the margin indicate the migration position of laminin, the 410 kDa species or A, B_1 and B_2 chains respectively.

laminin-expressing cell lines (Cooper *et al.*, 1981). The polyclonal antibody selects laminin, a 410 kDa and a 210 kDa component from the cellular extract (Fig. 2*a*, lane 1; Fig. 2*b*, lane 3). Only laminin and the 410 kDa component are found in the secretion medium, where they display an M_r slightly higher than those of their intracellular homologues (Fig. 2*a*, lane 2). The secretion medium lacks the free B-chain-like 210 kDa component, which is therefore not secreted. It contains, however, two other molecular species with apparent molecular masses of 250 kDa and 310 kDa, which are not detected in the immunoprecipitate of the cell content [Fig. 2*a*, lane 2 (arrowheads)].

The monoclonal anti-laminin immunoglobulin 4C12 immunoprecipitates laminin and a free 410 kDa component from both cell extract (Fig. 2*c*, lane 5) and secretion medium (result not shown), but fails to select the cellular 210 kDa component and the secreted 250 and 310 kDa proteins. Upon reduction and alkylation, the polyclonal and monoclonal immunoprecipitates migrate as A and B chains (Fig. 2*b*, lane 4; Fig. 2*c*, lane 9), in accordance with the fact that laminin, which represents much of the immunoprecipitated material, is a disulphide-dependent polymer of A and B chains. The B chains in the reduced monoclonal immunoprecipate migrate, however, as a distinct doublet of chains likely to be B_1 (210 kDa) and B_2 chains (205 kDa) (Fig. 2*c*, lane 9). In contrast, the B material in the reduced polyclonal immunoprecipitate migrates as a single broad band (Fig. 2*b*, lane 4). The difference is probably due to the co-migration in the polyclonal immunoprecipitate of the free B material (Fig. 2*c*, lanes 7 and 10) not selected by the monoclonal antibody.

As shown by immunoblot analysis, the monoclonal antibody interacts independently with the free 410 kDa component and laminin (Fig. 1*b*, lane 9), proof that these molecules share a common epitope and an indication that the free 410 kDa species immunoprecipitated by the monoclonal antibody might be an unassembled laminin 'building block'.

This result is corroborated by the finding that both laminin and 410 kDa species, separated by sucrose-gradient ultracentrifugation, are immunoprecipitated by the monoclonal antibody (Fig. 3*a*). The SDS/PAGE analysis, under reducing conditions, of the 410 kDa species immunoprecipitated from the sucrose-gradient fractions shows that it dissociates as B_1 and B_2 chains (Fig. 3*b*). It is, therefore, a disulphide-bond-dependent B_1B_2 complex.

This complex could not be distinguished from putative free A chain on SDS/PAGE, because the molecules display a similar molecular mass. Free-A-subunit production could, however, be analysed by sequential

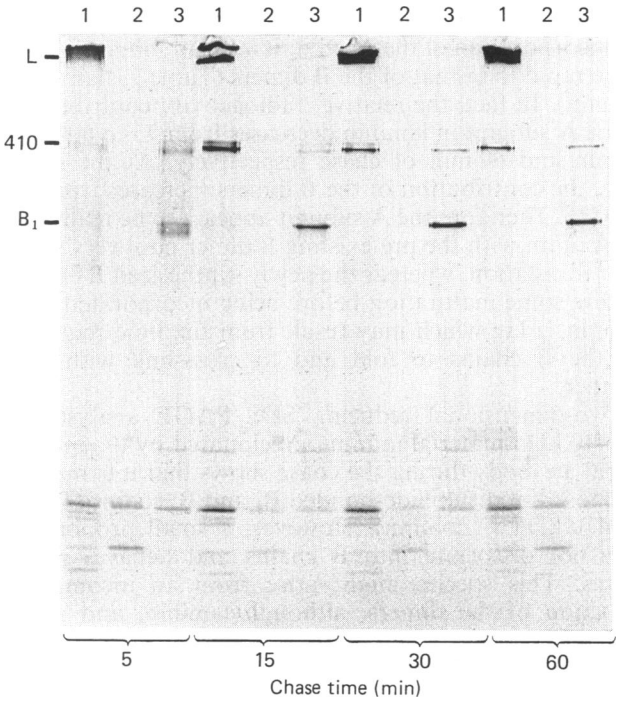

Fig. 4. SDS/PAGE analysis under non-reducing conditions of the material immunoprecipitated from the PFHR9 cell content during pulse–chase

The cell lysates of PFHR9 chased for 5, 15, 30 and 60 min, after a pulse labelling with [^{35}S]methionine were immunoprecipitated with a high-capacity anti-laminin monoclonal immunosorbent. The supernatant of the monoclonal immunoprecipitations was recovered and divided into equal fractions, which were immunoprecipitated again with monoclonal and polyclonal immunosorbents (sequential immunoprecipitations). The Figure is a fluorogram of an SDS/3–12%-gradient polyacrylamide gel loaded with: lanes 1, the monoclonal immunoprecipitates; lanes 2 and 3, the monoclonal (2) or the polyclonal (3) sequential immunoprecipitates. L, 410 and B_1 refer to the migration positions of laminin, the 410 kDa component and the B_1 chain respectively.

immunoprecipitation of the PFHR9 material first with the monoclonal antibody, which should precipitate most of the B dimers, leaving in the supernatant the free A subunits, which could then be immunoprecipitated by the polyclonal antibody. This procedure reveals that most of the laminin and the 410 kDa species in the cell extract is precipitated by the monoclonal antibody, leaving almost only the B_1-like 210 kDa protein free for immunoprecipitation by the polyclonal antibody (Fig. 2*c*, lanes 7 and 10). Therefore the intracellular pool of free A chains or unassembled B_2 chains appears to be extremely small, most likely because these molecules associate very soon after translation to form laminin or B dimers.

Pulse–chase analysis of laminin biosynthesis

The amount of radiolabelled material that could be immunoprecipitated from the PFHR9 cell contents after a pulse–chase increased rapidly, reaching a peak at 15 min after the chase. However, in contrast with the ^{35}S-labelled trichloroacetic-acid-precipitable material which peaked at the very beginning of chase, the amount of material immunoprecipitated after 5 min of chase was 50% lower than that precipitated later on, indicating that the early laminin products were inefficiently immunoprecipitated because of poor immunoreactivity, low extractibility or longer-than-average translation time. After 60 min of chase, the cellular material related to laminin decreased in proportion to the amount released into the culture medium. The release kinetics of newly synthesized laminin and radioimmunoassayable laminin appeared similar, and half of both of these cellular pools were released by 5 h (results not shown). Therefore, in the first 1 h of chase, any fluctuations in the levels of the material related to laminin are more likely to arise from biosynthetic process than from secretion.

Table 1. Newly synthesized laminin, 410 kDa and B_1 subunits: time course of production in PFHR9 cells

Lanes 1 and 3 in the fluorogram displayed in Fig. 4 were scanned. The absolute biosynthesis refers to the absorbances related to laminin (L) or 410 kDa (400) in lanes 1 and the absorbance (*A*) related to the free B_1 chains (B_1) in lanes 3. At a given chase time, relative biosynthesis equals to *A*(L) or *A*(400) multiplied by 100 and divided by *A*(L)+*A*(400).

Chase time (min)	Absolute biosynthesis			Relative biosynthesis (%)	
	L	400	B_1	L	400
5	43	4	12	90	10
15	43	29	16	60	40
30	67	17	18	80	20
60	72	11	13	87	13

The SDS/PAGE analysis of the unreduced monoclonal-antibody immunoprecipitate (Fig. 4, lanes 1) shows that the highest levels of newly synthesized laminin in cells are reached 1 h after the chase. Actually, 60% of this amount had already been produced by 15 min after the chase, indicating that the assembly of laminin from subunits proceeds rapidly (Table 1). The 410 kDa material peaks transiently 15 min after the chase and decreases rapidly thereafter, falling to 20% of the peak value by 1 h after the chase (mean value from six different pulse–chase experiments). Thereafter, the ratio of laminin to 410 kDa does not vary much, indicating little further transformation of this remaining 410 kDa fraction (result not shown). Most of the 410 kDa material is therefore processed simultaneously as laminin is forming, strongly suggesting a precursor–product relationship between these species.

The polyclonal antibody sequentially applied to the supernatant of the monoclonal immunoprecipitate selects little additional laminin (Fig. 4, lanes 3). More 410 kDa material is found; however, this species displays processing kinetics that are different from that of the monoclonally selected 410 kDa, being highest 5 min after chase and decreasing progressively thereafter. In addition, the polyclonal antibody selects substantial amounts of free B_1 chains, which increase progressively up to 30 min after the chase (Fig. 4, lanes 3, and Table 1). There is little free B_2 chain that appears during the course of chase. In fact, only small amounts of such a species

can be distinguished 5 min after the chase, where the free B material migrates diffusely [Fig. 4, lane 3 (chase time 5 min)].

The incorporation rate of the A, B_1 and B_2 chains in either laminin or the 410 kDa species was evaluated by SDS/PAGE analysis of the reduced and alkylated monoclonal-antibody immunoprecipitate. As shown in Fig. 5, by 15 min after the chase, 90% of the biosynthesized A and B_2 chains are already incorporated into laminin or the 410 kDa protein. As there is little or no free B_2 chain that appears during the course of chase, most of the B_2 chains formed during biosynthesis is therefore found combined to other B chains as free B dimers, or assembled into laminin, indicating that the B_2 chains associate very rapidly after translation. Similarly, after translation, the A subunit must associate very rapidly with the B chains to form laminin, because very little free A subunit appears during the chase (Fig. 5, lanes 3). In contrast, the incorporation of the B_1 chain is slower and less complete, since ultimately 50% of the B_1 chain produced will never become incorporated and thus will remain free (Fig. 5, lanes 3).

The dynamics of the incorporation of the B chains into laminin or the 410 kDa species could not be evaluated accurately because the B dimers, which are present in significant amounts early in the chase, dissociate to yield B chains upon reduction. Therefore laminin and the 410 kDa species had to be analysed separately by two-dimensional SDS/PAGE. The present study shows that the incorporation of the A subunit into laminin is slightly more rapid than that of the B dimers (Table 2, 'laminin' column). In fact, the relative radioactivity contribution of the A subunit in laminin decreases from 75 to 60% at 15 min and 60 min of chase respectively. At the same time, the contribution of the B dimers increases from 25 to 40%. Therefore the A subunit appears to be ready for association with the pre-existing B dimer pool very soon after translation, whereas the newly synthesized B chains require some maturation before being incorporated into laminin, a lag which may result from the time required for the B chains to fold and to cross-link with one another.

Two-dimensional reducing SDS/PAGE analysis of the 410 kDa material immunoprecipitated by the monoclonal antibody during the chase shows that it is mainly composed of disulphide-bonded B_1 and B_2 chains (Table 2, '410 kDa I' column). However, a small proportion does not dissociate into B chains and behaves as A chains. This species might arise from an incomplete reduction of the dimers, although laminin, and most probably also the B dimers, are very sensitive to reducing agents (Rao *et al.*, 1982). Alternatively, some A subunit might co-precipitate with the B dimers as a result of a high-affinity interaction between the subunits forming a complex resembling laminin but lacking interchain disulphide cross-links. Such a complex could be a short-

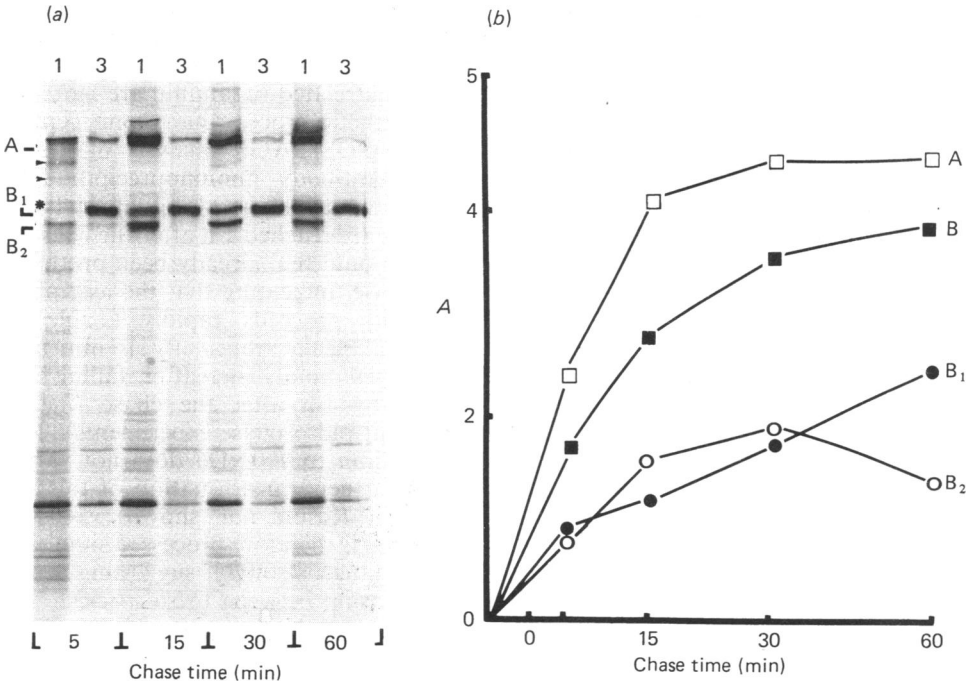

Fig. 5. SDS/PAGE analysis under reducing conditions of the material from the PFHR9-cell content immunoprecipitated during pulse–chase

The material analysed in Fig. 4 (lanes 1 and 3) was reduced and alkylated and electrophoresed on an SDS/3–12%-gradient-polyacrylamide slab gel. (*a*) Fluorogram of a gel loaded with the reduced monoclonal immunoprecipitates (lanes 1) and the reduced sequential polyclonal immunoprecipitates (lanes 3) from lysates of PFHR9 cells chased for 5, 15, 30 and 60 min after the pulse. A, B_1 and B_2 refer to the migration positions of these respective laminin subunits. (Arrowheads and the asterisk in track 1, chase time 5 min, point to putative precursors of the A and B_1 subunits respectively.) (*b*) Lanes 1 in (*a*) were scanned by densitometry, and the absorbances of the material related to the laminin A subunit (□), the B_1 subunit (●) and the B_2 subunit (○) were plotted as a function of chase time. B (■) is the sum of the absorbances related to B_1 and B_2.

Table 2. Dynamics of incorporation of the newly synthesized A, B_1 and B_2 laminin subunits into cellular PFHR9 laminin and the laminin-related 410 kDa molecular species

Laminin and the 410 kDa species in the monoclonal or sequential polyclonal immunoprecipitates from the cell content of PFHR9 chased for 5, 15, 30 and 60 min after the pulse, were purified by non-reducing electrophoresis on a 2% agarose gel (first dimension). Laminin in the electrophoresed monoclonal immunoprecipiates, 410 kDa I and 410 kDa II, corresponding to the 410 kDa species precipitated by the monoclonal antibody and the polyclonal antibody used in sequence to the monoclonal antibody respectively, were excised from their respective migration position in the first-dimension gel and further analysed after reduction and alkylation by SDS/3–12%-gradient-PAGE (second dimension). At any given chase time, the relative contribution of A, B_1 and B_2 to newly synthesized laminin, 410 kDa I and 410 kDa II could be evaluated by scanning the fluorograms of these second-dimension gels (results not shown). The absorbances related to the A, B_1 and B_2 were measured and are expressed as a percentage of the total absorbance in the scanned track. In some cases, the sum of $A+B_1+B_2$ is less than 100, owing to the presence of some material migrating between the A and the B_1 chains. The extensive analysis presented in the Table was verified by two additional independent experiments performed on chase-time −15 min and −60 min immunoprecipitates, which showed a similar dynamics of incorporation of A, B_1 and B_2 into laminin, 410 kDa I and 410 kDa II.

Chase time (min)	Relative contribution (%)								
	Laminin			410 kDa I			410 kDa II		
	A	B_1	B_2	A	B_1	B_2	A	B_1	B_2
5	47	22	0	52	23	16	47	29	20
15	75	7	13	28	29	31	53	28	8
30	67	12	13	22	35	30	31	68	0
60	58	23	12	10	44	29	11	88	0

lived biosynthetic intermediate, or might result from an association of the free subunits that occurs *in vitro* after cell lysis. Interestingly, after 30 and 60 min of chase, the 410 kDa species selected by the polyclonal antibody used subsequently to the monoclonal antibody (Table 2, '410 kDa II' column), dissociates mostly into B_1 chains upon reduction, indicating that a fraction of the B_1 pool can self-associate to form B_1 homodimers. However, the size of this fraction, which is small in comparison with that of the free B_1 chains, indicates that the propensity for B_1 to dimerize is low. The progressive enrichment in B_1 over B_2 that occurs during the chase in the monoclonal immunoprecipitate which encompasses laminin and B dimers (Fig. 5*b*), also can be observed without time lag in individual B dimers and laminin (Table 2, 'Laminin' and '410 kDa I' columns). This result indicates that the formation of the B dimer is limited by the availability of B_2 chains, probably a consequence of a transcription-related difference in the size of the B_1- and B_2-chain pools. It also indicates that some of the unassembled B_1 chains remain accessible for some time for associations with 'younger' B_2 chains.

Qualitative modifications during laminin biosynthesis

Short-lived molecular species appear at the earliest time of chase: these are (*a*) a faint doublet that appears in place of the B_1 chain (Fig. 5*a*, track 1, chase time 5 min), which could be the B_1a and B_1b precursors of B_1 (Kurkinen *et al.*, 1983) and (*b*) molecules migrating faster than the A chain (Fig. 5*a*: track 1, chase time 5 min), which could be precursors to this molecule (Durkin *et al.*, 1986). These molecules are not found free, but are already cross-linked with the other chains, indicating, if they are indeed precursors, that assembly does not require much post-translational modification, but is more a matter of protein sequence. In the later times of the chase, as a result of post-translational processing (Cooper *et al.*, 1981), both the A and B chains migrate at higher apparent-M_r positions (Fig. 5*a*), tending to the migration pattern of secreted laminin, where the A and B chains migrate diffusely (Fig. 2*c*, lane 11).

Localization of laminin by immunoelectron microscopy

The immunoelectron microscopy of the cell line labelled with the laminin monoclonal antibody reveals that most of the laminin detected is deposited on the outer side of the plasma membrane (Fig. 6). In rare instances some material could be detected in the cell cytoplasm, adjacent to the internal side of the cell plasma membrane. However, laminin could never be detected inside of the cells, stored as a secretory reserve or in the cellular apparatus. The same results were also obtained with the polyclonal antibody; they cannot be related to poor penetration of reagents due to cell-fixation artefacts, since various different intracellular antigens could be detected by using the same technique (Charpin *et al.*, 1986). The paucity of intracellular laminin is in contrast with the rather large amounts produced by the cells. Therefore, intracellular laminin is not detected either because it is associated with other antigens that decrease its immunoreactivity, or more likely because it is very rapidly exported after biosynthesis.

DISCUSSION

An anti-laminin monoclonal antibody, sensitive to the native conformation of laminin, immunoprecipitates both laminin and a 410 kDa species. The latter appears to consist of disulphide-linked laminin B_1 and B_2 chains. Since the monoclonal antibody is sensitive to the native conformation and does not interact with free B_1 chains, it is unlikely that it would precipitate a mixture of B_1 and B_2 homodimers; thus it appears that the 410 kDa species is a B_1B_2 heterodimer. Such a constitution for the molecule has been predicted by Barlow *et al.* (1984) and deduced from the primary structure of the *C*-terminal part of both B_1 and B_2 chains, determined by sequencing the DNA complementary to their respective mRNAs. According to these predictions, B_1 and B_2 fold to form α-helices that generate coiled-coil structures involving two or three strands of B chains or proteins presenting similar structural features (heptad repeats of hydrophobic amino acids). The finding, in PFHR9, of high amounts of free B_1 chains and low amounts of B_1 homodimers, indicates that the self-association of B_1 is not favoured. In contrast, chain interaction appears to be heterodimorphic, which provides an efficient folding of the chains and a stable structure.

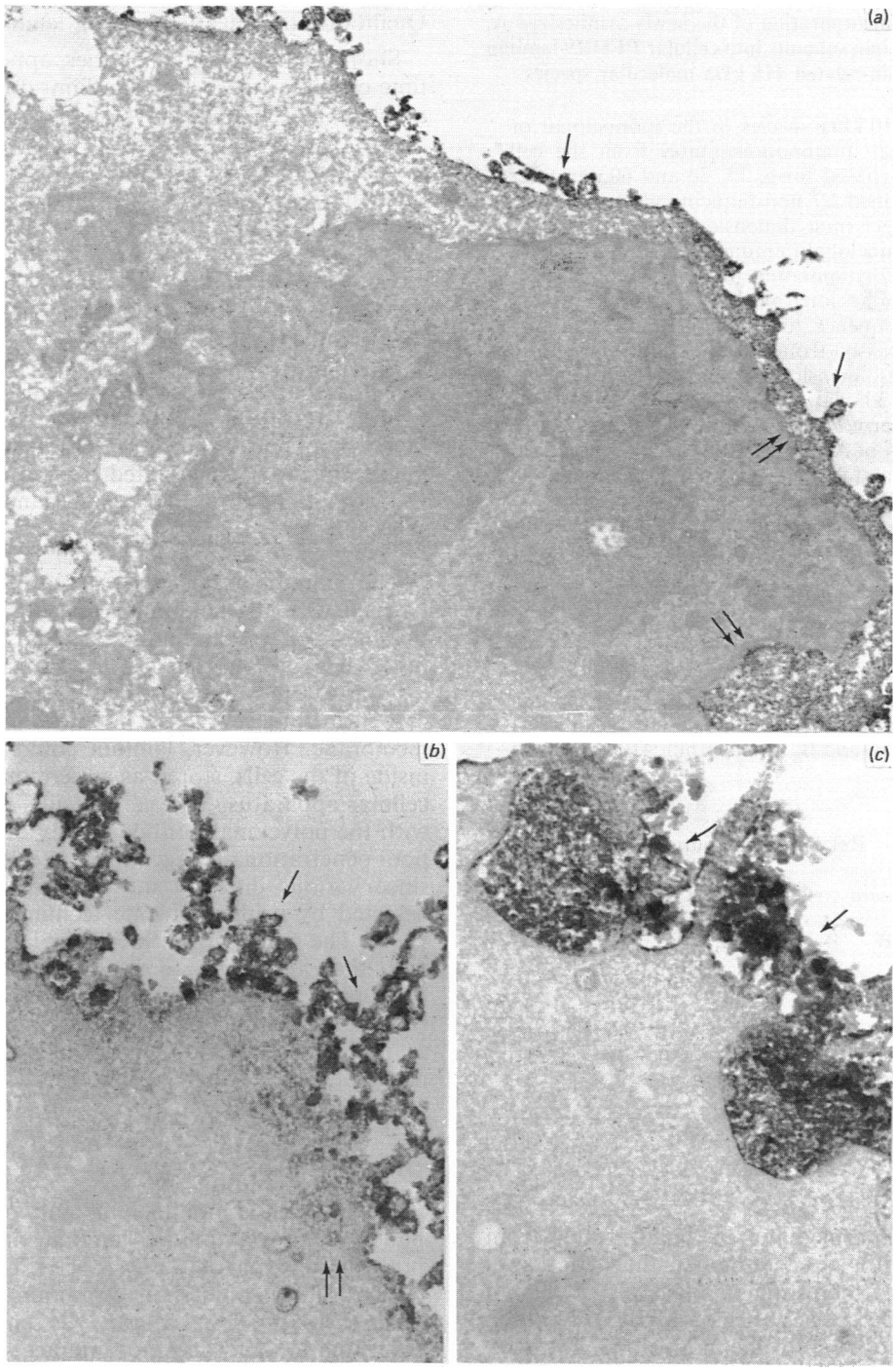

Fig. 6. Immunoelectron-microscopic detection with the monoclonal anti-laminin antibody 4C12 of pre-embedded PFHR9 cells with the avidin–biotin peroxidase technique

(*a*) Positive staining is mainly present on the outer side of the cell membrane (arrow) and in some cells only on the cytoplasmic side of the plasma membrane (double arrow) (magnification 76000 ×). (*b*) High magnification focused on the laminin-positive staining at the cell membrane and villi (arrow). Note a light staining of the cell cytoplasm (double arrows) (magnification 10100 ×). (*c*) Laminin staining is located on the cell membrane and appears as aggregates of excreted material (arrow) (magnification 12600 ×).

The formation of B dimers has already been described in the laminin-producing human choriocarcinoma JAR (Peters *et al.*, 1985) and the F9 teratocarcinoma induced to endodermal differentiation by retinoic acid and dibutyryl cyclic AMP (Morita *et al.*, 1985). Digestion fragments of EHS-sarcoma laminin consisting of disulphide-bonded B_1 and B_2 segments have also been isolated (Paulsson *et al.*, 1985). The formation of the B_1B_2 heterodimers appears, therefore, to be a general phenomenon in matrix-producing cells. In PFHR9, the formation of the dimer is limited by the B_2 production rate, since B_2 folds rapidly after translation and, in

contrast with B_1, never appears in free form. The relative expression of B_1 and B_2 in PFHR9 appears to be similar to that in the induced F9 cell line (Morita *et al.*, 1985), but differs from JAR (Peters *et al.*, 1985) and from uninduced F9 or the PYS cell line (Cooper *et al.*, 1981, 1983), where B_2 is produced in excess over B_1. However, as demonstrated in JAR, the B_1B_2 dimerization also proceeds rapidly and might be limited in this case by the B_1 production rate.

With respect to laminin, the dynamics of dimer biosynthesis are consistent with a precursor–product model. Furthermore, during pulse–chase experiments, the B_2/B_1 ratio decreases progressively and simultaneously in both the B dimers and laminin. The data indicate that some of the dimers associate rapidly with the A subunits to form laminin and suggest strongly that the incorporation of the B chains in laminin does not occur independently, but proceeds via the formation of intermediate dimers. About 60 and 40% of the [^{35}S]-methionine incorporated into PFHR9 laminin arise from the A and B chains respectively, values that best fit a model of laminin consisting of one A chain and two B chains. [It is known that methionine residues are evenly distributed in the chains (Sakashita & Ruoslahti, 1980)]. The levels of free A subunit during biosynthesis are extremely low; thus, unless they were greatly underestimated due to proteolysis, A chains associate extremely rapidly after translation with the available B dimers. As some of the B dimers remain unprocessed, the levels of the A subunit seem to control the laminin-production rate. Consequently, the pattern of laminin assembly appears similar in the JAR, F9, and PFHR9 cell lines, strongly suggesting that it is governed by an appropriate gene switching and by chain interactions involving rather high specificities. Cellular translocation away from the translation sites probably explains why the dimers and the B_1 subunits produced in excess to the A and B_2 chains respectively are not eventually assembled to a greater extent. The assembled B dimer is exported by PFHR9 as it is in the PYS cell line and mouse parietal endoderm (Cooper *et al.*, 1981). Its kinetics of release are similar to those of laminin, as indicated by a constancy in the laminin-to-B-dimer ratio in the secretion medium over periods of chase as long as 5 h (J.-C. S. Lissitzky, unpublished work). In contrast, the cellular free B_1 subunit is not released by the cell line, and it is not yet known whether the molecule is processed further to the 250 kDa protein found in the secretion medium or whether it is metabolized intracellularly.

Surprisingly, despite its high production rate in the PFHR9 cell line, laminin could not be detected within the cells, and most of the protein was found associated with the outside of the plasma membrane. This result is in accord with immunohistological studies of laminin in tissues, where the protein is usually found extracellularly, with only occasional cytoplasmic stainings. Lack of detection of intracellular laminin due to poor immunoreactivity of laminin subunits as they assemble to form the native molecule in a process linked to, or just preceding, secretion, can be excluded by the study of the laminin-biosynthetic features discussed above. Therefore, in the PFHR9 cell line, the protein appears to be exported very rapidly after it is produced. It can be forseen that these particular features of laminin biosynthesis and secretion will make it difficult to evaluate precisely the levels of the laminin expression using immunological methods, especially in tissues such as tumours, where the turnover of the extracellular laminin-like species cannot be evaluated.

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