Merosin is synthesized by thyroid cells in primary culture irrespective of cellular organization

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SUMMARY

The in vitro synthesis and deposition of laminin family glycoproteins were studied using primary porcine thyroid cells cultured as monolayers or in follicles. The latter organization mimics the in vivo state of these polarized epithelial cells. In both cell systems a trimeric molecule was immunoprecipitated by using polyclonal antibodies against EHS-laminin. When the cells were fully polarized the protein was found at the basal pole of cells, irrespective of their organization. However, this molecule was different from laminin purified from a traditional source, the murine Engelbreth-Holm-Swarm (EHS) tumor. Thyroid cell laminin was composed of two light chains, analogous to EHS B1 and B2, and a disulfide-bonded heavy chain not found in EHS-laminin. The heavy chain was first synthesized as a 380 kDa polypeptide, then rapidly cleaved to a doublet of 350-380 kDa, which was subsequently found in both cell extracts and conditioned culture media. This thyroid laminin variant was compared with merosin, another variant found in the basement membranes of trophoblast, Schwann cells, striated muscle and liver. The heavy chain (M) of merosin shows homology to EHS-laminin heavy chain at the C-terminal domain, and is usually found as two polypeptides of 80 kDa and 300 kDa (Ehrig K., Leivo I., Argraves W. S., Ruoslahti E. and Engvall E. *Proc. Nat. Acad. Sci.* 87, 3264-3268, 1990). mRNA of the M chain was identified by RT-PCR in freshly isolated thyrocytes as well as in 6-day-old cultured thyroid cells. Furthermore, both the classical laminin heavy chain and the 350 kDa variant were detected by immunoblotting and immunofluorescence in the thyroid gland in vivo. All these results suggest strongly that merosin is a basement membrane component of thyroid cells in vivo and in vitro.

Key words: thyroid, extracellular matrix, merosin, epithelial polarity

INTRODUCTION

Basement membranes represent specialized structures of extracellular matrices that separate epithelial or endothelial cells from interstitial connective tissue; they also surround many cell types such as muscle, nerve and fat cells (Vracko, 1978; Leblond and Inoue, 1989). The major constituents of these structures include type IV collagen (Kühn et al., 1985), laminin (Beck et al., 1990; Martin and Timpl, 1987), entactin (Carlin et al., 1981; Paulsson et al., 1986), and heparan sulfate proteoglycans (Hassell et al., 1985). It is thought that basement membranes control tissue compartmentalization, cell differentiation and migration, and the selective filtration of macromolecules (Farquhar, 1991; Yurchenco and Schittny, 1990).

Laminin (800 kDa) is a large and complex molecule that was first isolated from the murine Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979). This molecule is composed of three genetically distinct subunits: two light chains of about 200 kDa each, designated B1 and B2, and one 400 kDa A heavy chain (Engel et al., 1981). The B1, B2 and A chains are connected by disulfide bonds in a cross-shaped molecule (Martin and

Timpl, 1987). Laminin has diverse effects, including stimulation of cell growth and differentiation, promotion of neurite outgrowth, and cell adhesion, spreading and motility (reviewed by Timpl and Dziadek, 1986; Schittny and Yurchenco, 1989). The large size of this molecule gives it the potential to span the basement membrane; its multidomain structure allows the molecule to mediate the interactions of multiple basement membrane components, and to interact with various receptors at cell surfaces. The cellular activities of laminin are presumably mediated by these cell surface receptors, many of which belong to the integrin family (Mecham, 1991). Although widely distributed, laminin is less ubiquitous than was first thought, and variants are present in other basement membranes. Among these variants, merosin is different in that a heavy M chain replaces the A chain and the molecule has a restricted pattern of expression (Engvall et al., 1990; Engvall, 1993).

The influence of culture conditions on cell architecture and differentiation has been well characterized with epithelial cells such as thyroid cells, Madin-Darby canine kidney (MDCK) cells (Montesano et al., 1991), or mammary cells (Barcellos-

Hoff et al. 1989). For example, thyroid cells in primary culture exhibit different cell organizations and degrees of expression of thyroid functions depending on the substratum and the presence of thyrotropin-stimulating hormone in the medium (Fayet et al., 1971; Mauchamp et al., 1979; Inoue et al., 1980; Chambard et al., 1981). In contrast, little is known about how the cell organization influences the secretion of extracellular matrix components. Because thyroid cells in vitro range from monolayers to follicles, we used these cells for an in vitro study of the effects of cellular organization on the synthesis and deposition of basement membrane components and, more precisely, glycoproteins of the laminin family.

Previous studies have shown that in culture there is no basement membrane organization when thyroid cells are cultured alone (Fayet et al., 1971; Cau et al., 1976; Remy et al., 1977). Nevertheless, they synthesize type IV collagen (Wadeleux et al., 1985), heparan sulfate proteoglycans (Giraud and Bouchilloux, 1983) and fibronectin (Giraud et al., 1981). In the present investigation, we have established that isolated porcine thyroid cells cultured either in monolayers or in follicles were able to synthesize and deposit a laminin variant. This variant was a trimer of two light chains, analogous to B1 and B2 chains, and one heavy chain of 380 kDa that was rapidly cleaved and then detected as a doublet of 350-380 kDa. We identified this molecule as merosin by several approaches. It is a novel form in which heavy chain M processing differs from that in other tissues.

MATERIALS AND METHODS

Isolation and culture of thyroid cells

Thyroid glands from freshly killed pigs were collected at the slaughterhouse and kept on ice until dissection. Muscle, fat and thyroid capsule were removed aseptically. Thyroid cells were dissociated by successive trypsin (Sigma) and EGTA treatments at 37°C according to the method of Fayet et al.(1970) modified by Mauchamp et al.(1979). Cells were then plated on plastic dishes in Dulbecco's modified essential medium (DMEM) (Gibco BRL, Cergy Pontoise, France) supplemented with glucose (1 g/liter), 10% newborn calf serum, penicillin (200 i.u./ml) and streptomycin (50 µg/ml). For formation of follicles, thyrotropin-stimulating hormone (TSH) at 1 munit/ml was added at the onset of culture. Gelatin was observed to stabilize follicular organization and was added at 0.5%. The patterns of laminin deposition were found to be unchanged by the addition of gelatin. Thyroid cells were seeded into 35 mm diameter dishes (monolayers: 4×10^6 cells/dish; follicles: 6×10^6 cells/dish), at a final volume of 2 ml. The culture medium was renewed every 2 or 3 days.

PFHR9 cell line culture

The mouse laminin-producing cell line of the endodermal type, PFHR9 (Chung et al., 1977), was a generous gift from Dr J. C. Lissitsky (CNRS, Lyon, France). The cells were grown to confluency in DMEM, 10% fetal calf serum.

Antisera

Rabbit anti-laminin sera were produced by injection of laminin purified from EHS tumor cells by anion-exchange chromatography (Timpl et al., 1979). This mouse laminin was a generous gift from Dr J. C. Lissitsky (Lyon, France). The specificity of mouse laminin antiserum was assessed in two ways: immunoblotting against EHS-secreted material produced only two bands whose molecular masses correspond to the A and B1-B2 chains of laminin (see Fig. 4B, below);

immunofluorescence on thin frozen sections of pig thyroid showed a positive laminin immunostaining surrounding thyroid follicles, corresponding to a continuous basal lamina (see Fig. 1A, below).

Polyclonal and monoclonal antibodies against the M chain of human merosin as well as monoclonal antibodies against the A chain of human laminin were kindly provided by Dr E. Engvall (Cancer Research Foundation, La Jolla, CA). Mouse monoclonal antibodies against rabbit aminopeptidase N (APN) were a generous gift from Dr S. Maroux (CNRS, Marseille, France).

Tissues and cells treatments

Porcine thyroid glands were cut into 1-2 mm blocks and then either fixed in formaldehyde (for laminin and APN detection) or quick-frozen in liquid nitrogen (for merosin detection).

The formaldehyde fixation was performed at 4°C in 4% formaldehyde in phosphate buffered saline (PBS) for 1 hour. After PBS washes, samples were incubated for 10 minutes in 50 mM NH_4Cl in PBS, then in 5% sucrose in PBS for 1 hour, followed by 20% sucrose in PBS overnight. Frozen tissues were sectioned at 5 µm in a cryostat (Microm H.M 500).

Since we observed that M chain antigenicity was lost after formaldehyde fixation, samples of porcine thyroid gland and tongue were quick-frozen in liquid nitrogen and sectioned as described before. Sections were attached to polylysine-coated glass slides, air dried and fixed in acetone at −20°C for 5 minutes.

Cells cultured on plastic dishes were washed three times with PBS, then fixed in 4% formaldehyde for 20 minutes at 4°C and washed three times in PBS. When needed, cells were permeabilized by incubation for 4 minutes in PBS-0.2% Triton X-100 at room temperature.

Immunohistochemistry

Cells or sections were incubated for 10 minutes in 50 mM NH_4Cl in PBS, then twice for 5 minutes each in PBS containing 4% (w/v) of bovine serum albumin (buffer A) to minimize nonspecific labelling. Then, the following immunocytochemical reactions were performed at room temperature in buffer A: (a) 1 hour incubation with a mixture of rabbit laminin antiserum (1/50) and mouse anti-aminopeptidase N IgG (20 µg/ml) for double immunolocalization; (b) four 5 minute washes; (c) incubation for 20 minutes with biotinylated sheep anti-mouse Ig (1/50; Amersham); (d) washes as in (b); (e) incubation with a mixture of fluorescein-conjugated swine immunoglobulins to rabbit immunoglobulins (1/40; Dako, Copenhagen, Denmark) and sulforhodamin-streptavidin (1/100; Boehringer Mannheim Diagnostics) for 20 minutes. Finally, cells or sections were given four 5 minute washes in buffer A, mounted with a glass coverslip in glycerol/PBS, 9/1 (v/v), containing 1 mg/ml phenylenediamine (Eastman, Rochester, NY) (Johnson et al., 1981), and examined in a Zeiss Photomicroscope III (Carl Zeiss, Inc., New York, NY) equipped with a III RS epicondenser. Monoclonal antibodies against A and M heavy chains were used from culture supernatants diluted 1:2. The specificity of the antisera was verified by the absence of staining with preimmune serum and with secondary antibodies alone.

Cell labelling and immunoprecipitation

Cultured cells were either pulse-labelled for 2 hours or metabolically labelled under steady-state conditions for 18 hours with 0.25 mCi/ml or 0.1 mCi/ml L-[^{35}S]methionine (1000 Ci/mmol; Amersham), respectively, on day 6 after plating. At the end of labelling, media were removed and centrifuged to eliminate cellular debris. Supernatants were adjusted to immunoprecipitation conditions: 50 mM Tris-HCl, 0.4 M NaCl, 2 mM EDTA, 1% Triton X-100 (w/v), 0.4% SDS (w/v), 0.1 mg/ml ovomucoid, 1 µg/ml aprotinin, 1 mM PMSF, 2 mM β-phenylpropionate, pH 8 (buffer I). Cells were scraped into buffer I (520 µl/dish), homogenized extensively and the insoluble material was removed by centrifugation at 16,000 g for 30 minutes at 4°C in an Eppendorf centrifuge. Fractions

were supplemented by L-methionine to a final concentration of 6 mM and stored at −70°C until use. Samples of 150 µl of cell extracts and 350 µl of media were used for immunoprecipitation. The samples were cleared with 50 µl of rabbit preimmune serum (overnight incubation, shaking, 4°C), then treated for 2 hours with 75 µl of Protein A-Sepharose (Sigma) adjusted to a final volume of 500 µl with buffer I containing 4% BSA (w/v). After removal of the beads by centrifugation for 5 minutes at 16,000 **g**, the supernatants were incubated with 50 µl rabbit anti-mouse laminin for 3 hours followed by 75 µl Protein A-Sepharose slurry for 2 hours. The immunoprecipitation of newly synthesized laminin was quantitative under these conditions.

Sodium dodecyl sulfate-gel electrophoresis and autoradiography

After immunoadsorption, the antigen-antibody complexes were washed seven times with buffer I, twice with 50 mM Tris-HCl buffer (pH 8.8), then solubilized and treated as previously described (Bartles et al., 1987). Proteins from buffer I extract were precipitated with TCA (Hubbard et al., 1985). The resulting pellets were washed with 90% acetone, 10% M HCl to remove the detergents and then solubilized as immunoprecipitates. All samples were applied to 3% to 12% polyacrylamide gels as described (Blobel and Dobberstein, 1975). Proteins were stained with Coomassie Blue R 250 (Fluka). For autoradiography, gels were dried and exposed at −70°C to Hyperfilm-βmax (Amersham).

[14]C-labelled proteins standards (Gibco BRL, Cergy Pontoise, France) were included in the gels (1.12 nCi/lane). These were myosin heavy chains, 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; and lysozyme, 14.3 kDa.

Immunoblotting

After separation by SDS-PAGE, polypeptides were transferred to nitrocellulose (BA 83, Schleicher and Schuell) overnight at 50 V (constant voltage) in a buffer containing 20% methanol, 20 mM Tris and 150 mM glycine, pH 8. Nonspecific protein binding sites were blocked with a 30 minute incubation in PBS containing 10% bovine serum. Immunodetection was carried out by incubating for 1 hour with a 1/1000 dilution of the polyclonal antibodies against mouse laminin or human merosin. The bound antibodies were visualized by using a 1/1000 dilution of biotin-conjugated donkey anti-rabbit Ig (Amersham) for 40 minutes followed by ^{125}I-streptavidin, 0.5 µCi/ml (20-40 µCi/µg; Amersham). All the dilutions and the washing steps were in PBS containing 10% bovine serum and 0.2% Triton X-100. Autoradiography was performed at −70°C with intensifying screens and Hyperfilm-MP (Amersham).

Isolation of laminin and merosin from thyroid gland

Laminin and merosin were extracted from thyroid essentially as described by Paulsson et al. (1991) for bovine heart. Briefly, pig thyroid glands were collected rapidly after killing. Following dissection as described for cell isolation, the tissue was quick-frozen in liquid nitrogen and stored at −70°C. After thawing, 10 g samples were extracted with 50 ml of 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, with proteinase inhibitors and then with Tris buffer containing 10 mM EDTA. The EDTA extracts were precipitated with ammonium sulfate (40% saturation); this step eliminated most of the thyroglobulin. The precipitate was dialyzed against 50 mM ammonium bicarbonate, pH 8.0, and applied to a column (3 ml) of heparin-agarose (Sigma) in the same buffer (Engvall et al., 1992). The adsorbed proteins were eluted with 0.5 M NaCl, 50 mM ammonium bicarbonate, pH 8.0. The fractions were submitted to TCA precipitation, and laminin and merosin were detected following SDS-PAGE and immunoblotting with respective sera.

Reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) and Southern blotting

Total RNA was prepared from freshly isolated porcine thyroid cells and 6-day-old cultures, when cells had organized into follicles. RNA was treated with RQ1 RNase-free DNase (Promega), and reverse transcription was performed with 3 mg RNA, the avian murine virus reverse transcriptase (AMV-RT; Promega) and primers for the M chain DNA antisense strand. The cDNA was amplified with Pfu polymerase (Stratagene) for 30 cycles of denaturation for 45 seconds at 93°C, annealing for 30 seconds at 55°C and extension for 90 seconds at 74°C in an Omnigene Hybaid apparatus. The two pairs of primers used for the amplification were: for the M chain sense strand, 5′-AAAGTATCTGTGTCTTCAGGA-3′; for the M chain antisense strand, 5′-AGTGAATGTAATCACACGTACAGC-3′ (Engvall et al., 1992); the internal primers used for the nested amplification were: for the M chain sense strand, 5′-CTGGCTATAGAAATGCGTAAA-3′; and for the M chain antisense strand, 5′-TCTCACA-GAAATAGTTCCATT-3′. The calculated size of the PCR product is 156 bp. Product (10 µl) was analyzed on a 3% agarose gel and transferred to nylon membrane (hybond N+; Amersham). The blot was prehybridized and hybridized in a buffer containing 6× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7), 2× Denhardt's solution (100× Denhardt's solution is 2% Ficoll, 2% polyvinylpyrrolidone, 2% bovine serum albumin), 1% SDS and 0.4 mg/ml denatured salmon sperm DNA. Hybridization was carried out for 20 hours at 48°C by using the ^{32}P-labelled internal primer M chain antisense strand. The blot was washed twice for 15 minutes in 2× SSC, 0.1% SDS at 42°C then exposed for 15 to 30 minutes to Hyperfilm MP (Amersham). The size of the cDNA was estimated using a 1 kb ladder (BRL).

RESULTS

Laminin antibody stains the basal pole of thyroid cells in vivo and in culture

Thyroid follicles in vivo rest upon a thin, continuous basement membrane (Heimann, 1966; Klinck et al., 1970). As shown in Fig. 1A, in thin frozen sections of epithelial tissue this basement membrane was stained with a rabbit polyclonal serum raised against EHS-laminin. Aminopeptidase N (APN), which is a transmembrane glycoprotein that is restricted to the apical plasma membrane of these cells (Feracci et al., 1981), was used as an indicator of the degree of cell polarity. Its labelling was restricted to the membrane abutting the colloid in vivo (Fig. 1B), while laminin labelling was on the basement membrane at the basal pole of the cells (Fig. 1A).

Since we were interested in the relationship of basement membrane components to cell polarity, we cultured isolated thyroid cells in two different organizations: monolayers and follicles. In monolayers, all the cells have their apical poles accessible to the culture medium. In follicles, which have an organization that mimics the situation in vivo, the apical poles are inaccessible and confined to the inside of the follicles (Fig. 2). Although we already knew that isolated thyroid cells cannot organize a visible basement membrane when cultured by themselves, we wanted to determine whether they expressed and deposited laminin.
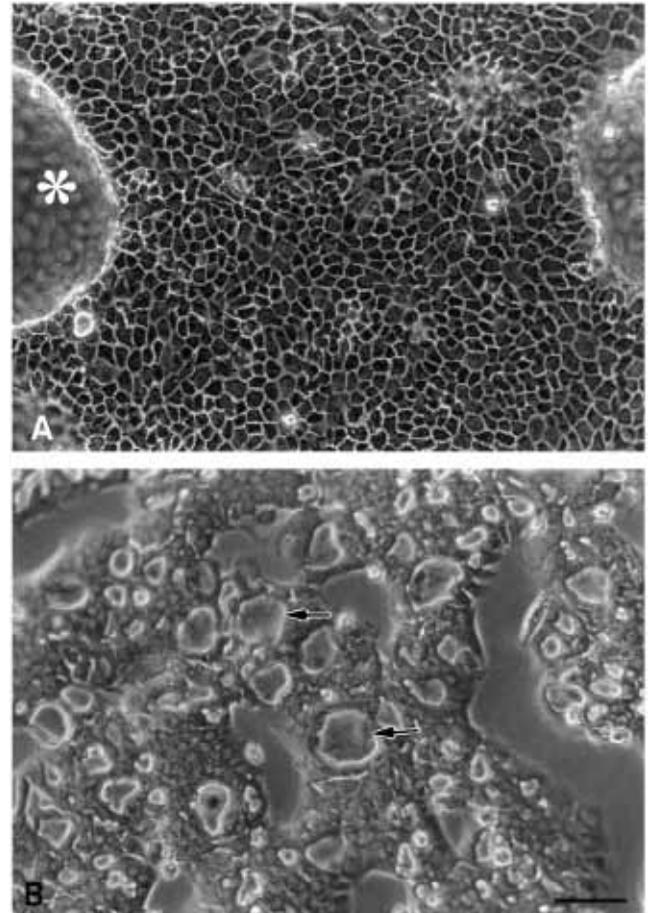
We first looked at cultured cells whose organization was already established, a process that took 6 days for either type of organization. Immediately after isolation by successive trypsin-EGTA treatments, the cells had lost all polarity (Tixier-Vidal et al., 1969; Cau et al., 1976; Feracci et al., 1981). They
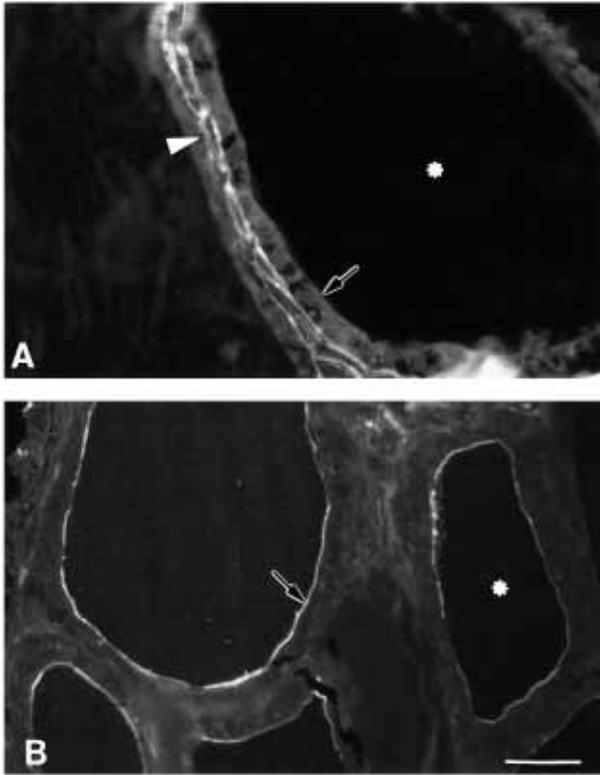
Fig. 1. Immunohistochemical localization of (A) laminin, and (B) aminopeptidase N in thin frozen sections of porcine thyroid tissue. Laminin was stained with a anti-EHS-laminin serum, and APN with a mouse monoclonal antibody. Laminin can be seen on basement membranes (arrowhead) surrounding two follicles in (A), while APN marks the apical poles of the thyroid cells (arrow) in four different follicles in (B). Asterisks indicate the follicular lumens. Bar, 25 μm.


Fig. 2. Phase-contrast microscopy of thyroid cells after 7 days of culture without (A) or with (B) thyrotropin present from the onset of culturing. (A) The cells are organized in a tight monolayer, forming domes (asterisk). (B) The cells in the presence of the hormone reassociate into three-dimensional structures showing the typical morphology of thyroid gland follicles. Follicular lumens, smaller than the ones in vivo, are indicated by arrows. Bar, 60 μm.

subsequently attached to plastic dishes within 12 hours and formed a tight monolayer by 6 days, as evidenced by domes (Fig. 2A) and were stable for at least 4 weeks. At 6 days, APN was restricted to the apical pole of the cells and showed a punctate staining pattern due to microvilli (Fig. 3A; Feracci et al., 1981). Laminin was arranged as clusters between some cells, without any well-defined network organization (Fig. 3B). No intracellular labelling was observed. The need for membrane permeabilization to visualize laminin indicated that probably the molecule was deposited either onto the plastic dish or at least on the basolateral side of the cells. Any attempt to remove the cells while keeping laminin on the dish was unsuccessful, confirming that laminin was not organized in a network. Without membrane permeabilization, there was no staining with laminin antibodies (not shown).

When TSH was added at the onset of culture, the cells organized into closed follicles that had morphological and functional properties similar to those observed for thyroid follicles in vivo (Hovsepian et al., 1982). This organization was maintained for at least 2 weeks. From 6-12 days of culture, the apical marker APN was present in rings that clearly marked the inner surface of the follicles (Fig. 3C). APN could only be visualized after detergent treatment. Laminin was associated with the same follicles, but on the opposite side to APN and

in a network of long fibers (Fig. 3D). It was visualized without detergent permeabilization.

In light of laminin's localization in fully organized cultures, we examined the formation of laminin networks in younger cultures.

After 2 days of culture, the monolayer was not yet fully organized as evidenced by the APN staining (Fig. 3E). In some cells, fluorescence appeared selectively at the apical pole of cells. In other cells not yet organized into a tight monolayer, APN remained randomly distributed over the entire surface. This localization of APN was somewhat different from that observed previously (Hovsepian et al., 1982), possibly due to different cell substrates and/or culture conditions. In the same cells, Fig. 3F shows that laminin was present both intracellularly and in a very thin cobweb organization. It was not possible to determine the relative intra- and extracellular proportions of laminin because cells were mostly aggregated and the staining intensity was very weak. This staining needed membrane permeabilization to be detected.

In cells seeded and cultured for 2 days in the presence of
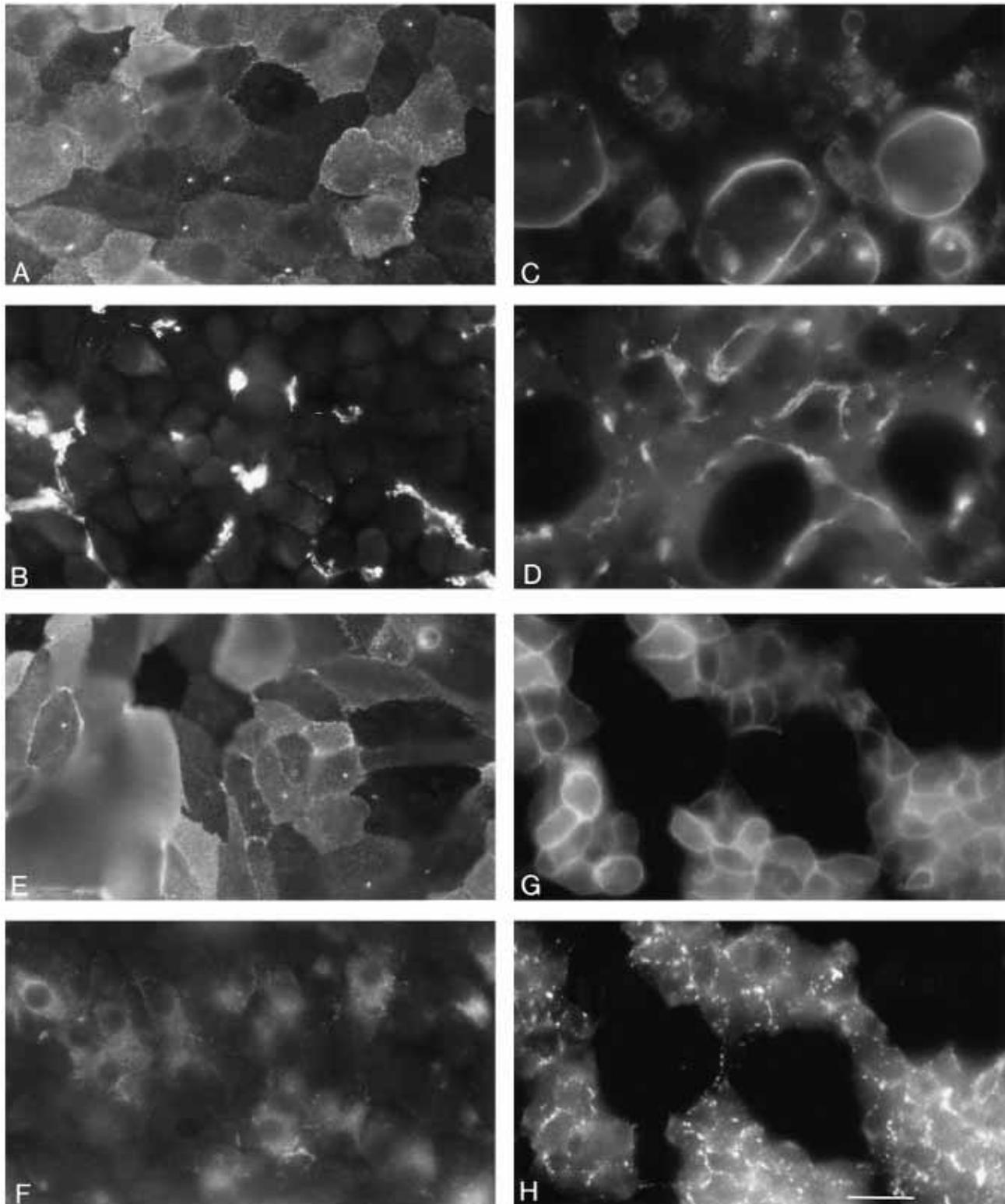
Fig. 3. Laminin deposition in primary cultures of porcine thyroid cells organized in monolayers (left panels) or in follicles (right panels) after 12 days (A-D) and 2 days (E-H) of plating. Laminin and APN were co-stained with a rabbit antiserum against EHS-laminin (B,D,F and H) and a monoclonal antibody to APN (A,C,E and G), which marked the apical plasma membrane domain. (A-D) After 12 days of culture the monolayer was fully organized and laminin formed foci or patches at the basal face of cells (A, B). On cells in follicular organization, laminin was distributed in a fibrillar pattern surrounding follicles (C,D). Detergent permeabilization was needed in B and C to obtain these labellings; the same patterns were obtained in A and D with or without membrane permeabilization. (E-H) On the 2nd day of culture the monolayer was still in formation (compare with E and A) and a very faint positive fluorescence for laminin, which needed detergent permeabilization, was observed (F). On TSH-supplemented cultures, when APN was still all over the plasma membranes (G), laminin presented a bright punctate distribution (H). The same labelling was obtained in G and H with or without membrane permeabilization. Bar, 16 μm.

TSH, APN was present all over the cell surface just as after cell isolation. Laminin was also detected around the cell surface and exhibited a punctate pattern that co-localized with APN (Fig. 3F,H). Both molecules were equally accessible to their antibodies with or without membrane permeabilization.

Taken together, these results indicated that, after cell organization was established, laminin was present at the basal side of thyroid cells in culture irrespective of their organization. However, the patterns of deposition were consistently different in monolayers and follicles, reflecting differences in cellular organization and suggesting a difference either in the expression of laminin receptors or in the molecular arrangement of laminin.

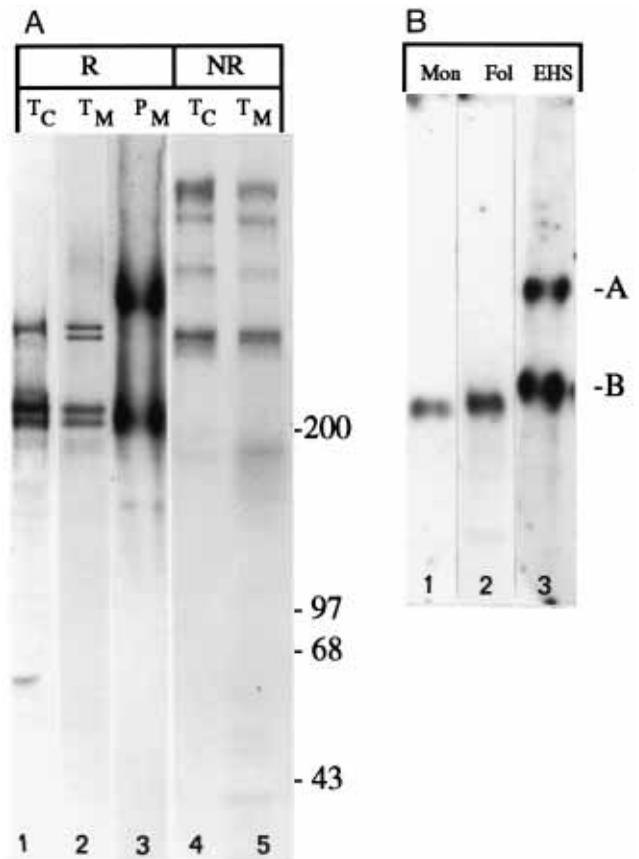## Thyroid cells in culture synthesize a laminin isoform

Was there new synthesis of laminin by isolated thyroid cells? By western blotting, no laminin could be detected in freshly isolated cells (not shown). This result was not surprising, given the trypsin-EGTA treatments required for cell isolation, the known sensitivity of laminin to proteinases (Ott et al., 1982) and the efficiency with which EDTA extracts laminin (Paulsson et al., 1987). The amount of laminin deposited by thyrocytes obviously increased between 2 and 12 days of culture, at least when cells were organized in follicular structures (Fig. 3). These observations were confirmed by western blotting analysis (not shown) and strongly suggest that the cells were actively synthesizing laminin.

For further confirmation, we immunoprecipitated ^{35}S-labelled laminin from lysates of cell monolayers or follicles after a 2 hour labelling with [^{35}S]methionine. Under reducing conditions, three polypeptide chains were detected in cells organized in follicles (Fig. 4A, lane 1); an identical result was obtained with cells organized in monolayers (not shown). Interestingly, the immunoprecipitates contained laminin B1 and B2 chains plus a third band migrating at ~380 kDa, which is faster than the A chain of EHS-laminin, at a molecular mass ~400 kDa (compare lanes 1 and 3, Fig. 4A). About 10% and 30% of ^{35}S-laminin was recovered in the conditioned medium from monolayers and follicles, respectively. Analysis of laminin immunoprecipitated from conditioned medium from monolayers and follicles after a chase or after labelling under steady-state conditions showed a doublet of 350-380 kDa for the laminin heavy chain (Fig. 4, lane 2). This doublet also appeared in immunoprecipitates from cell extracts metabolically labelled under steady-state conditions (not shown). Since only the 380 kDa heavy chain was present after 2 hours of labelling it is possible that the 350 kDa polypeptide resulted from proteolytic cleavage of the 380 kDa chain.

In order to confirm the difference observed in electrophoretic mobilities between A chain and the heavy chain of the thyroid laminin variant, we performed similar experiments using a mouse endodermal cell line PFHR9 (Chung et al., 1977). Lissitzky et al. (1988, 1992) have described the synthesis by PFHR9 cells of laminin identical to the form produced by EHS tumor. A laminin immunoprecipitation experiment from PFHR9 cell-conditioned medium was run in parallel with the thyroid samples (Fig. 4, lane 3). The difference in electrophoretic mobility between the two heavy chains confirmed our observation described above. In the immunoprecipitates from thyroid cell extracts B1 and B2 chains were present in higher amounts than the heavy chain doublet, sug-

gesting the presence of some B1-B2 complexes devoid of heavy chain (Fig. 4A, lane 1).

The homology between the molecule precipitated from the cell and that from the medium was confirmed by analysis of these immunoprecipitates on SDS-PAGE under nonreducing conditions, and the same pattern was observed (Fig. 4A, lanes 4 and 5). We also detected a band at about 400 kDa, which is

Fig. 4. Thyroid cells synthesize a laminin variant. (A) Follicles 6 days after plating were labelled for 2 hours (for cell extracts), or under steady-state conditions for 18 hours (for media), with [^{35}S]methionine. Immunoprecipitates of cell lysates (Tc, lane 1) and conditioned media (Tm, lane 2) were analyzed on 3% to 12% gradient SDS-PAGE under reducing conditions. Samples were precipitated with anti-EHS-laminin serum. The same immunoprecipitation experiment was performed with PFHR9 cell-conditioned medium after labelling under steady-state conditions (Pm, lane 3). In lanes 4 and 5, respectively, laminin immunoprecipitates of thyroid cell lysate (Tc), and medium (Tm) after metabolic labelling were analysed under nonreducing conditions. The positions of radiolabelled size markers (in kDa) are indicated in the margin. For the calculation of the heavy chain molecular mass, thyroglobulin (330 kDa) and the laminin A heavy chain (400 kDa) were used in addition to these standard molecules. (B) Immunoblot analysis of 80 µg proteins of monolayer (Mon, lane 1), follicles (Fol, lane 2) cell extracts, and 0.6 µg EHS tumor cells extract (EHS) with rabbit anti-EHS-laminin serum. Laminin A and B chains detected in EHS tumor cell extract are noted in the margin. The difference in migration between the B chains (lanes 1-2 and 3) may be due to the different amounts of protein loaded. It should be noted that ^{125}I-streptavidin labelling was the only way a positive signal could be obtained by western blotting on these cell extracts.

probably the B1-B2 complex. Other bands with slower electrophoretic mobility were detected, which probably corresponded to different combinations of the protein chains. Because of the differences in mobility between reduced and nonreduced proteins, and the nonlinearity in this part of the gel between migration and molecular mass, we have not pursued further the identification of these bands.

To characterize this laminin heavy chain variant in greater detail, we immunoblotted cell extracts of monolayers and follicles with the same rabbit EHS-laminin antiserum as was used for the immunoprecipitation experiments. After transfer onto nitrocellulose the EHS-laminin rabbit antiserum recognized all three chains of the mouse laminin, but only the light chains (unresolved in this experiment) in the extracts from cultured thyroid cells (Fig. 4B). The heavy chain of the thyroid laminin variant with its unusual molecular mass was not recognized by this serum.

The fact that we detected two chains by immunoblotting and three chains by immunoprecipitating this molecule could be explained by the techniques. In immunoprecipitation experiments, owing to the covalent association between the three chains, the heavy chain most probably coprecipitates with the light chains and therefore is not recognized by the antibodies. After denaturation for SDS-PAGE analysis and western blotting, the dissociation of the three polypeptides precedes antibody recognition and each chain is detected individually.

Laminin variants in the thyroid gland

The absence of detection by western blotting of the heavy chain synthesized by porcine thyroid cells in culture might have been due to lack of interspecies cross-reactivity for the heavy chains of porcine and mouse laminins. To test this hypothesis, we submitted porcine thyroid tissue to EDTA extraction and ammonium sulfate precipitation following a procedure described by Paulsson and Saladin (1989) for selective extraction of mouse heart laminin. The extract was adsorbed on heparin-agarose. The salt-eluted molecules were subjected to SDS-PAGE under reducing conditions, blotted onto nitrocellulose, and incubated with anti-EHS-laminin serum. Thyroid extracts prepared this way had both laminin light chains *and* a third chain with the same electrophoretic mobility as the A chain of laminin secreted by the EHS tumor (Fig. 5A, lanes 1 and 2). This result clearly indicated that the porcine laminin heavy chain was recognized by the anti-EHS-laminin serum. Therefore the failure to detect the heavy chain of the thyroid laminin variant with this serum (Fig. 4B) was *not* due to lack of interspecies crossreactivity. The presence of laminin heavy chain A in the thyroid gland was confirmed by labelling of tissue sections with monoclonal antibody specific for the human laminin A chain (Fig. 5B). The protein was localized in basement membranes surrounding follicles and some blood vessels.

We next tried different antibodies against human merosin (Leivo and Engvall, 1988; Ehrig et al., 1990). We tested seven monoclonal antibodies by immunofluorescence on cryosections of porcine tongue, where merosin is present (Leivo and Engvall, 1988). Two monoclonal antibodies, 4F11 and 1B4, gave a positive interspecies crossreactivity and stained basement membranes that surrounded striated muscle fibers, as described by Leivo and Engvall (1988). On porcine thyroid tissue, these two monoclonal antibodies gave the same

basement membrane-restricted positive reaction, as illustrated in Fig. 5C, demonstrating expression of merosin in the thyroid gland. In some cases, we also detected labelling of the basolateral membrane in thyrocytes. Therefore both A and M chains were colocalized in the basement membranes that surround thyroid follicles.

By immunoblot analysis, a rabbit serum against a merosin peptide (Ehrig et al., 1990) stained a polypeptide of about 350 kDa in thyroid gland extracts (Fig. 5A lane 3). This polypeptide had the same electrophoretic mobility as the 350 kDa heavy chain immunoprecipitated from cell-conditioned media or from cell extracts after labelling under steady-state conditions (Fig. 4A).

Merosin is synthesized by thyroid cells in culture

All attempts to detect the merosin heavy chain in cell extracts or conditioned media by the same techniques as used for the tissue were unsuccessful. By immunoblot analysis, the amount of proteins required for detection was so high that the appropriate region of the gel was overwhelmed by thyroglobulin, the major protein secreted by these cells at 330 kDa.
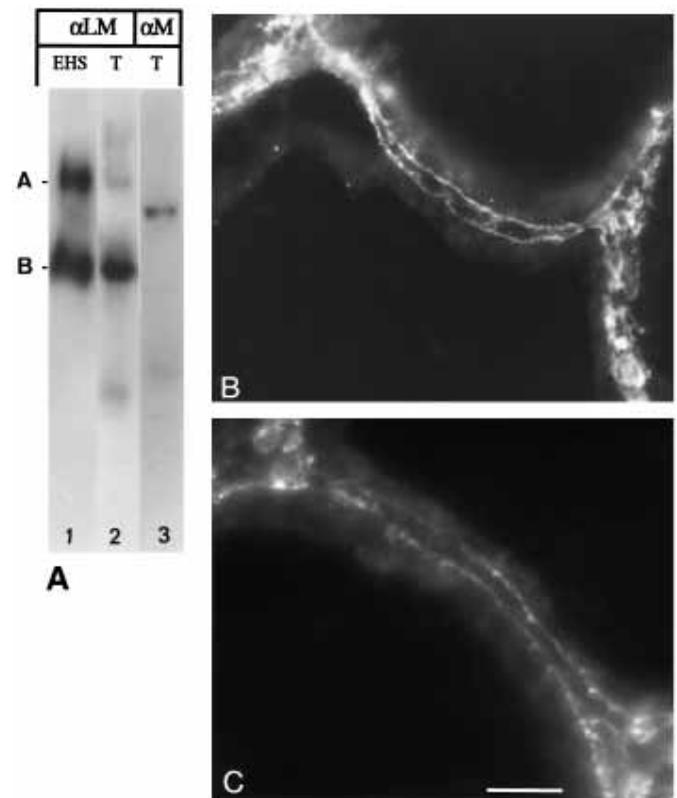
Fig. 5. A and M heavy chains are detected in the thyroid gland. (A) Thyroid gland extracts eluted from heparin-agarose were submitted to 3% to 12% gradient SDS-PAGE, then transferred to nitrocellulose (T, lanes 2 and 3). Blot was stained with anti-EHS-laminin serum (lane 2), and with a merosin peptide antiserum (lane 3). For comparison, an EHS tumor extract was electrophoresed in parallel and stained with anti-EHS-laminin (EHS, lane 1). (B,C) Distribution of A chain and M chains in thyroid gland. Both the monoclonal antibodies against A (B) and M (C) chains stained basement membranes lining follicles. Bar, 16 μm.
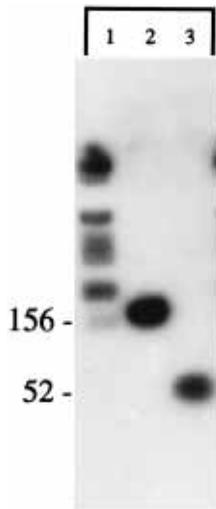
Fig. 6. RT-nested PCR analysis of merosin mRNA. RNA was extracted from porcine thyroid cells 6 days after plating with TSH (i.e. organized into follicles). The RNA was reverse transcribed, amplified with merosin primers and analysed by the Southern blotting method. Lane 1, molecular mass markers in bp; lane 2, PCR product detection by the labelled internal primer M chain antisense strand; lane 3, same as lane 2 but after digestion with *Ban*I.

We performed immunofluorescence on acetone-fixed cells, since the antibodies did not react with formaldehyde-fixed cryosections. We were able to detect fiber structures on cells organized in follicles as with the EHS-laminin antiserum. Nonetheless, with this fixation procedure we could not eliminate a very high background, even with secondary antibody alone (results not shown). We turned to detection of merosin M chain mRNA using the sensitive RT-nested PCR method followed by Southern blot analysis. The first pair of primers used represented sequences of M chain cDNA (Engvall et al., 1992). The pair of internal primers were chosen inside the amplified cDNA sequence with the view that they might be more M-chain-specific. The expected 156 bp PCR product was obtained when mRNA from 6-day-old porcine thyroid cells organized into follicles was reverse transcribed and amplified with the merosin primers (Fig. 6, lane 2). Its size was identical to that calculated from human placental cDNA (Ehrig et al., 1990). The same result was obtained using mRNA from freshly isolated thyroid cells (not shown), indicating the presence of M chain transcripts both in the gland and in thyrocytes in culture.

For further information on the specificity of the PCR product, we digested the 156 bp fragment with the restriction enzyme *Ban*I, which has only one site in the defined merosin M chain. In a 3% agarose gel, we detected two ethidium bromide-stained bands of 104 bp and 52 bp, respectively, as predicted by analysis of human placental merosin cDNA (not shown). Only the 52 bp band is detected on the autoradiogram, because hybridization was performed with only the M chain internal antisense strand primer (Fig. 6, lane 3). This positive reaction indicated that the sequence of the M chain mRNA amplified is conserved across species. Taken together these results showed that the laminin variant synthesized by thyrocytes was the intact form of the merosin heavy chain (380 kDa), which could be cleaved in a 350 kDa chain found in the gland.

DISCUSSION

In this study we found that thyroid cells cultured either in monolayers or in follicles are capable of producing a laminin variant, merosin. The molecule was deposited on the basolateral side of the cells even without ultrastructural evidence of basement membrane organization. The different cellular organizations that we have examined did not influence these phenomena.

Serum raised against laminin extracted from EHS tumor reacted positively at the basal plasma membrane domain of thyroid cell cultures in both monolayers and follicles. Normal pig thyroid sections were also positive with this serum. These observations indicated that laminin was present at the basal pole of thyroid cells in vivo as well as in culture. Immunoprecipitation of newly synthesized molecules clearly indicated that a trimeric molecule was being made by these cells in both culture systems. This molecule was in fact a laminin variant in which B1 and B2 light chains are disulfide bonded to a 350-380 kDa heavy chain doublet. This heavy chain doublet replaced the A chain in the laminin of thyroid cell cultures.

Laminin is a considerably more complex molecule than previously thought and, recently, several variants have been described. Variants that lack the classical heavy chain A have been reported, including laminin from rat RN22 Schwanoma (Davis et al., 1985; Edgar et al., 1988) and 3T3 adipocytes (Aratani and Kitigawa, 1988). Recently two new members of the laminin chain family have been characterized: one with a truncated form of the B2 chain (Kallunki et al., 1992) and the other with a 190 kDa molecule substituted for the A chain (Marinkovich et al., 1992). Both have been found in different subsets of epithelial basement membranes. S-laminin (synapse-associated laminin) contains, with normal A and B2 chains, a variant chain S that shows 52% amino acid sequence identity to the B1 chain (Hunter et al., 1989). This s-laminin is expressed at synaptic sites in muscle, at the perineurium and in some blood vessels (Sanes et al., 1990).

Merosin heavy chain (M) is usually detected as two polypeptides of 300 and 80 kDa (Ehrig et al., 1990). Expression of M chain transcripts was demonstrated by polymerase chain reaction in 6-day-old cultured thyroid cells. The only molecule of the laminin family synthesized by these cells had a heavy chain that appeared just after its synthesis as a 380 kDa chain, then as a doublet of 350-380 kDa. Expression of M chain transcripts was also detected in thyroid cells just after isolation from the tissue. In immunofluorescence experiments, the thyroid gland basement membrane was stained by an antibody directed against human M chain, and a 350 kDa band was detected by immunoblot of tissue extract. Taken together, these results strongly indicated that the M chain of merosin and the thyroid 350-380 kDa heavy chains were related and most probably homologous to the same protein purified from different species. We suggest that the M chain usually detected as two polypeptide chains (300+80 kDa) was observed here for the first time in its intact, uncleaved form, which then underwent different processing. These different molecular forms of merosin probably have different three-dimentional conformations that could modulate the biological properties of the protein.

Merosin is restricted to the trophoblast basement membrane of placenta, striated muscle and Schwann cells (Ehrig et al., 1990; Leivo and Engvall, 1988; Paulsson and Saladin, 1989; Paulsson et al., 1991). The M chain is also expressed transiently in neonatal liver and in adult liver after partial hepatectomy, in association with B1 and B2 or with S and B2 chains

(Wewer et al., 1992). We have shown that this molecule is present in basement membranes surrounding thyroid follicles. To our knowledge, the cellular origin of laminin variants has never been studied in the thyroid gland, and we have detected the presence of two laminin isoforms, classical laminin and merosin, in basement membranes that surround each follicle (Heimann, 1966). In this tissue, the basement membrane, together with the junctional complexes between the follicular epithelial cells, might have an additional biological role, since it seems to prevent leakage of thyroglobulin into the perifollicular space. Different cell types are present and many of them could be involved in the synthesis of the basement membrane lining the follicles. In addition to the principal cells of the follicles, there is a smaller population of cells, designated as C cells, which synthesize calcitonin. Morphological studies indicate that C cells are not involved in basement membrane formation during embryogenesis, the follicular basement membrane being constituted before C cells appearance (Stoeckel and Porte, 1970). The interfollicular spaces contain numerous capillaries, occasional fibroblasts, and small bundles of collagen fibrils. Endothelial cells are possible sources of the classical laminin with an A chain (Gospodarowicz et al., 1981; Sanes et al., 1990). The possibility that some fibroblastic cells are responsible for synthesis of merosin in culture can be excluded by considering the pattern of laminin deposition after 2 days of culture under TSH stimulation where each thyrocyte was decorated by punctate labelling with laminin antibodies (Fig. 3H). The results presented in this paper demonstrate the synthesis of merosin by isolated thyrocytes. Moreover, the A chain was not detected by immunofluorescence in the cultured cells, confirming that there is no A chain synthesis by isolated thyrocytes (not shown). Nonetheless, both A and M chains were detected in basement membranes lining follicles, while the two chains are not usually present together in the same basement membranes (Engvall, 1993). All of these laminin variants have probably evolved for expressing different biological functions, but the nature of these functions is not known.

Merosin expression occurred early during the establishment of both monolayers and follicles. It was observed as early as the second day of culture and was particularly prominent in follicles. Addition of TSH to established monolayers apparently does not affect the merosin pattern (not shown). This is in good agreement with previous studies (Giraud et al., 1981; Giraud and Bouchilloux, 1983; Wadeleux et al., 1985), indicating that the synthesis of fibronectin, total fraction of proteoglycans and type IV collagen by thyroid cells in primary culture do not seem to be influenced by this hormone.

There was strict polarity of deposition of merosin when the thyroid cell surface polarity had been established. In both systems, merosin was deposited on the basal side of the epithelial cells, and not on the apical surfaces. This is probably related to the efficacy of cell polarity for secretion and to the putative presence of specific receptors on the basal side of the plasma membrane. Moreover, a substantial fraction of merosin was detected in conditioned medium from follicles. This leaking may be due in culture to the absence of a physical barrier, which in vivo is constituted by the basement membrane network.

Little is known about the mechanisms involved in the assembly of the basement membrane components. Differenti-

ated porcine thyroid cells in primary culture have been shown to synthesize fibronectin (Giraud et al., 1981), heparan sulfate proteoglycans (Giraud and Bouchilloux, 1983) and type IV collagen (Wadeleux et al., 1985). With the additional synthesis of merosin presented in this paper, these cells should have the major components known to structure a basement membrane. Nevertheless, previous studies have shown that formation of basement membrane requires either cellular cooperation (Alquier et al., 1979) or additional basement membrane components (Garbi and Wollman, 1982; Espanet et al., 1992). Taken together these data could suggest that all these components are not synthesized by normal thyroid cells in the correct molecular ratio to elaborate a basement membrane structure identifiable by electron microscopy. Moreover, from neither of the immunoprecipitates from thyroid cell cultures (cell extract and conditioned medium) shown in Fig. 4A, did we observe the presence of entactin, a glycoprotein of basement membranes in complex with laminin (Paulsson et al., 1987). This protein is involved in the scaffolding of laminin and type IV collagen (Fox et al. 1991). Recently the absence of entactin was shown to parallel the absence of basement membrane in the liver (Wever et al., 1992). Ehrig et al. (1990) have described the same cross-shaped structure and some sequence homology between merosin and laminin. Therefore it will be interesting to determine if these molecules can be substituted in the basement membrane network. These thyroid cell culture systems could be useful to test if entactin is critical for basement membrane formation and/or stabilization.

Leivo and Engvall (1988) previously suggested that merosin plays a specific functional role associated with a high level of differentiation in tissues that express it. They indicated the absence of this molecule from 28 rat and human cell lines and its first detection, in tissues where it appeared, only after birth. Moreover, Garbi et al. (1988) have studied synthesis of basement membrane components by FRTL5 cells, a cell line of rat thyroid that has retained many specific thyroid functions and showed that these cells express laminin. The observation that primary cultures of normal thyroid cells exclusively express merosin strongly argues that merosin expression is related to a highly differentiated phenotype.

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