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Merosin, a protein specific for basement membranes of Schwann cells, striated muscle, and trophoblast, is expressed late in nerve and muscle development

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ABSTRACT **We have identified a tissue-specific basement membrane-associated protein by using monoclonal antibodies prepared against a protein fraction of human placenta. In immunofluorescence, the monoclonal antibodies stained basement membranes of Schwann cells, striated muscle, and trophoblast, whereas no reaction was seen with any other basement membrane or tissue structure. In antibody-affinity chromatography of proteolytic digests of human placenta, a 65-kDa polypeptide was bound by these monoclonal antibodies. Rabbit antisera and monoclonal antibodies raised against the isolated 65-kDa polypeptide stained human and monkey tissues identically to the original monoclonal antibodies and reacted with an 80-kDa polypeptide in tissue extracts prepared without proteolysis. The 65-kDa and 80-kDa polypeptides were shown to be immunologically distinct from laminin, type IV collagen, fibronectin, and major serum proteins. They presumably represent a novel basement membrane-associated protein, which we have named merosin. No merosin immunoreactivity could be detected in cultures of any of 28 established cell lines. In developing mouse tissues, merosin staining first appeared at the newborn stage. The restricted tissue distribution and late developmental appearance of merosin suggest that the protein has a tissue-specific function associated with a high level of differentiation.**

Basement membranes are sheets of extracellular matrix separating epithelial and other parenchymal cells from connective tissue stroma. They are thought to be important in development and tissue repair by promoting attachment, migration, and proliferation of cells (1, 2) and by mediating signals for tissue interactions (3–5). Molecular constituents common to all basement membranes include laminin (6, 7), type IV collagen (8), entactin (9), and heparan sulfate proteoglycan (10). Studies with monoclonal and polyclonal antibodies indicate that there are also tissue-specific antigens associated with the basement membrane zone (11–18). These include acetylcholinesterase in basement membranes of the neuromuscular junctions (17), complement fragment C3d bound to basement membranes of the glomerulus and placenta (15), and collagen type VII located subjacent to the basement membrane (18). Unidentified antigenic epitopes with a tissue-specific basement membrane distribution may belong to yet-unknown molecules or to isotypes of known molecules, or they may reflect diversity in the supramolecular assembly of known components (16). Identification of new basement membrane components with restricted tissue distribution could provide important tools for the study of tissue-specific functions of basement membranes in developing and adult tissues. In this paper, we report the identification of a basement membrane-associated protein specific for basement membranes of Schwann cells, striated muscle, and trophoblast.

MATERIALS AND METHODS

Monoclonal Antibodies and Rabbit Antisera. Monoclonal antibodies were produced against proteins extracted from human placenta as described (19). Hybridomas were screened by immunofluorescence of human and monkey tissues. The selected monoclonal antibodies, 4F11 and 1B4, were of the IgG1(κ) class and stained identically all tissues tested. Second-generation monoclonal antibodies were raised against reduced and alkylated 65-kDa polypeptide. Rabbit antisera were raised against the native 65-kDa fragment and affinity-purified by adsorption to and elution from 65-kDa fragment coupled to Sepharose.

Immunohistochemistry. Human placentas were obtained from cesarean sections or normal term deliveries. Other human tissues were obtained from autopsy. Newborn monkey (*Macaca fascicularis*) tissues were from the Primate Research Center (Alamogordo, NM). Mouse tissues were obtained from adult and embryonic BALB/c mice. In mouse embryos, the day of the vaginal plug was designated as day 0 of pregnancy. Deliveries took place on day 21. The tissues were quick-frozen in liquid nitrogen and 6-μm sections were made, air-dried, and fixed in cold acetone.

For immunofluorescence, sections were treated for 2 hr with hybridoma culture medium diluted 1:2, ascitic fluid diluted 1:100–500, or affinity-purified rabbit antibodies at 10–30 μg/ml, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) at 1:40 dilution as described (20). Inhibition of the binding of monoclonal antibodies to tissue sections was carried out by pretreating the sections with rabbit antiserum (at 1:10 dilution) or purified antibodies overnight and subsequently staining with the monoclonal antibodies followed by a mouse IgG-specific FITC conjugate.

In some experiments, tissue sections were digested with one of the following: trypsin (type III-S, Sigma), 100 μg/ml in phosphate-buffered saline (PBS: 0.10 M NaCl/0.05 M phosphate, pH 7.2) for 1 hr (21); pepsin (Cooper Biomedical, Malvern, PA), 4 mg/ml in 10 mM HCl for 1 hr at 37°C as described for fixed tissues (22); chondroitinase ABC (Sigma), 2500 units/ml in 50 mM Tris·HCl, pH 8.0/50 mM NaCl/50 mM sodium acetate for 3–24 hr at 37°C; or heparitinase (Seikagaku Kogyo, Tokyo), 1 unit/ml in 50 mM Tris·HCl, pH 7.0/5 mM calcium acetate for 3 hr at 37°C in the presence of protease inhibitors.

Isolation and Electrophoresis of the 65-kDa Fragment. The IgG fraction of 4F11 ascitic fluid was precipitated with 18%

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(wt/vol) Na_2SO_4, redissolved in 0.1 M $NaHCO_3$, and coupled to cyanogen bromide-activated Sepharose (Pharmacia). Placental extracts subjected to antibody-affinity chromatography included 4 M NaCl-precipitated fractions of a neutral salt extract (19), mild pepsin digests prepared as described (23), and chymotrypsin digests (150 μg of chymotrypsin per g of washed tissue). The tissue extracts were prerun through plain Sepharose and gelatin-Sepharose and then applied to the 4F11 antibody column. The column was washed with PBS and 1 M NaCl, and bound material was eluted with 1 M acetic acid or 4 M KSCN. For some experiments the protein was further purified by FPLC using a Mono Q column (Pharmacia). Protein preparations were analyzed by $NaDodSO_4$/PAGE using 2–16% or 4–30% gradient gels (Pharmacia).

Enzyme Immunoassays, Immunoblotting, and Immunoprecipitation. Wells of polystyrene microtitration plates (Flow Laboratories) were coated with the isolated 65-kDa fragment, human laminin (19), rat laminin (24), rat tail tendon type I collagen (25), mouse type IV collagen (Bethesda Research Laboratories), human type VI collagen (26), human plasma fibronectin (27), or bovine serum albumin, and bound antibodies were detected by ELISA as described (28). For studies on interactions between the various matrix proteins, microtitration wells were coated with one protein, the ligand was added in PBS with 0.05% Tween 20 for 2 hr at 37°C, and bound ligand was detected with the respective antiserum as above.

Immunoblotting was done using monoclonal antibodies to the reduced and alkylated 65-kDa fragment. Pieces of tissue were minced in PBS with 1 mM phenylmethylsulfonyl fluoride and then extracted with PBS containing 0.05% Tween 20 and 1 mM phenylmethylsulfonyl fluoride. The residue was boiled in $NaDodSO_4$/PAGE sample buffer and the $NaDodSO_4$-extracted proteins were separated by $NaDodSO_4$/PAGE and transferred electrophoretically to nitrocellulose. Merosin was detected on the nitrocellulose by ELISA using peroxidase-labeled anti-mouse IgG (23).

Metabolic labeling of cell cultures was carried out with [^{35}S]methionine and culture media were subjected to immunoprecipitation as described (15). The following cell lines were from American Type Culture Collection and other sources: A204, A431, BeWo, Caski, CRL-1573, FL, Hep G-2, HT-1080, HUVEC, IMR-32, IMR-90, JAR, JEG-3, L6, M-21, MB 8387, MB 9812, MCF-7, MG-63, MRC-5, PA-1, RD, RN-22, Rugli, SKLMS-1, SKGIIIa, U-373MG, WI-38, and 5838.

RESULTS

Localization of a Tissue-Specific Basement Membrane Antigen. Two monoclonal antibodies prepared by immunization with placental extracts, 4F11 and 1B4, showed a highly tissue-specific basement membrane-associated reactivity. These antibodies gave a bright fluorescence in the trophoblast basement membranes of term placenta (Fig. 1*a*), whereas no staining was seen in the fetal capillary basement membranes of chorionic villi (Fig. 1*a*) or in the basement membranes of the amnion membrane or the umbilical cord (data not shown). Basement membranes that surround striated muscle fibers in tongue and other skeletal muscles were stained brightly. This was particularly clear in tongue tissue at the pointed ends of those muscle fibers that attach to the dermis (Fig. 1*c*). Moreover, bright staining was seen in the Schwann cell basement membranes of peripheral nerves, whereas the endoneurium and the perineural sheath were negative (Fig. 1*e*). No staining was seen in the epithelial basement membrane of the tongue (Fig. 1*c*) or skin or in the walls of arteries, capillaries, or venules of these tissues (Fig. 1 *c*, *e*, and *g*). The epithelial and endothelial basement membranes in kidney (Fig. 1*k*), liver, colon (Fig. 1*i*), and bladder were also negative. Finally, smooth muscles of vessel walls, colon, and bladder were negative. No fluorescence was seen in cerebral or cerebellar cortices.

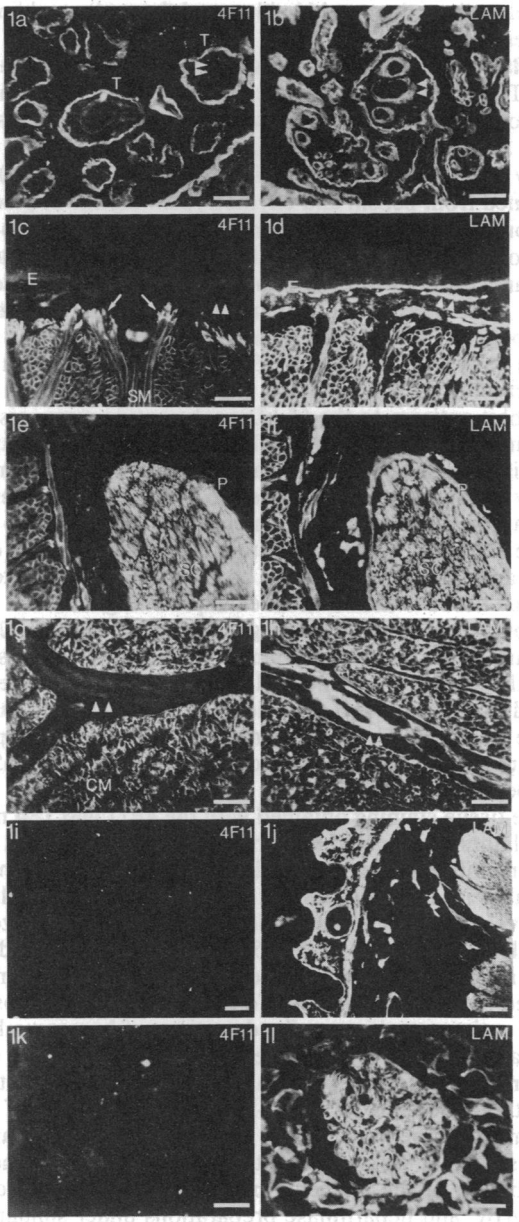

FIG. 1. Comparison of the tissue distributions of the 4F11 antigen and laminin (LAM) by immunofluorescence. In term placenta (*a*), the 4F11 antibody stains trophoblast (T) basement membranes. In tongue (*c*) and peripheral nerve (*e*), 4F11 fluorescence is seen in skeletal muscle (SM) basement membranes and Schwann cell (SC) basement membranes but not in the epithelial (E) basement membrane of the tongue or in the perineurium (P). Note the particularly intense 4F11 fluorescence in the pointed ends of muscle fibers that insert into dermis (*c*; arrows). In heart (*g*), 4F11 fluorescence surrounds cardiac muscle cells (CM). No fluorescence, however, is present in the smooth muscle wall of colon (*i*) or in the kidney (*k*). In all tissues, the 4F11 antigen is absent from vascular walls (arrowheads in *a*, *c*, and *g*), whereas laminin is present at these sites (arrowheads in *b*, *d*, and *h*) and in all basement membranes in all tissues. Sections in *a*, *b*, *k*, and *l* were from human tissues, and in *c–j* from monkey tissues. (Bars = 25 μm.)

In contrast, the tissue distribution of laminin, shown for comparison (Fig. 1 *b*, *d*, *f*, *h*, *j*, and *l*), includes all epithelial and endothelial basement membranes; smooth muscle of

vessel walls, colon, and bladder; and the perineural lining of nerves.

Isolation of a Protease-Resistant 65-kDa Polypeptide Antigen. To identify the antigen recognized by the 4F11 and 1B4 antibodies, various extracts and digests of human placenta were analyzed by antibody-affinity chromatography. Relatively large amounts of protein (4–6 mg per placenta) were obtained from pepsin and chymotrypsin digests of placenta. In contrast, negligible amounts of protein bound to 4F11 antibodies from several neutral salt extracts of placenta prepared without the use of proteases. The protein isolated from both pepsin and chymotrypsin digests migrated in $NaDodSO_4$/PAGE under nonreducing conditions as a major sharp polypeptide band at 55 kDa and at 65 kDa after reduction (Fig. 2).

Rabbit antisera to the 65-kDa polypeptide were raised by immunization with excised gel bands or with highly purified protein, and antibodies were affinity-purified by chromatography on Sepharose conjugated to the 65-kDa polypeptide. When the affinity-purified antibodies were used to stain tissue sections, a pattern of staining identical to that of the 4F11 and 1B4 antibodies was observed in all tissues tested. Tongue tissue is shown as an example in Fig. 3. If the tissue section was pretreated overnight with a low dilution (1:5) of the antiserum or affinity-purified antibodies and subsequently stained with the 4F11 monoclonal antibodies followed by a mouse IgG-specific FITC conjugate, no fluorescence was seen. These experiments confirm that the epitope recognized by the 4F11 antibodies is present in the 65-kDa polypeptide.

The tissue staining obtained with the 4F11 and 1B4 monoclonal antibodies could have represented a ubiquitous basement membrane component, the determinants of which were available in some tissues but not in others. It is unlikely, however, that all the antigenic determinants recognized by polyclonal antibodies to the 65-kDa polypeptide would have been masked in those basement membranes that were not stained by these antibodies. To exclude further the possibility of antigen masking, unfixed sections of human umbilical cord and placenta were treated with pepsin, trypsin, chondroitinase ABC, or heparitinase and then fixed and stained with the antibodies to the 65-kDa fragment. None of these enzyme treatments changed the distribution of staining, suggesting that the observed staining pattern reflects the true distribution of the antigenic protein. The same chondroitinase ABC and heparitinase preparations under similar conditions removed glycosaminoglycan side chains from chondroitin sulfate and heparan sulfate proteoglycans (29).

Characterization of the 65-kDa Polypeptide. Enzyme immunoassays were used to study the immunological identity of the 65-kDa polypeptide. Affinity-purified antibodies were added to microtitration wells coated with the 65-kDa polypeptide or with various extracellular matrix proteins. The antibodies reacted strongly with the 65-kDa polypeptide, whereas negligible or no reaction was observed with rat or human laminin, mouse type IV collagen, human fibronectin, or bovine serum albumin (Fig. 4). Thus, in addition to its unique tissue distribution, the 65-kDa polypeptide is also immunologically distinct from the tested basement membrane proteins.

Analysis of the amino acid composition of the 65-kDa polypeptide showed predominantly hydrophilic amino acids in proportions common to most proteins (data not shown). Hydroxyproline and hydroxylysine were absent, suggesting that the polypeptide does not contain collagenous domains. No amino sugars were observed in this analysis.

Interactions of the native 65-kDa polypeptide with molecules of the extracellular matrix were tested. Binding assays with extracellular matrix proteins were carried out both with the 65-kDa polypeptide in solid phase and addition of soluble ligands, or with ligands in the solid phase and addition of soluble 65-kDa polypeptide. In these assays, no interactions between the 65-kDa polypeptide and human or rat laminin, fibronectin, type IV collagen, or type VI collagen were observed. Similarly, there was no significant binding of the soluble 65-kDa polypeptide to heparin-Sepharose.

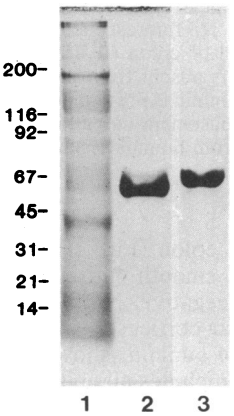

FIG. 2. $NaDodSO_4$/PAGE of the protein fraction obtained from a placental pepsin digest by affinity chromatography on monoclonal antibody 4F11. Polypeptides are separated in a 4–30% gradient gel and detected by Coomassie blue staining. Lane 1, polypeptides of the pepsin digest used for fractionation; lane 2, polypeptides bound to 4F11 antibody and analyzed unreduced; lane 3, polypeptides bound to the 4F11 antibody and analyzed after reduction with 2-mercaptoethanol. Positions and sizes (kDa) of standard proteins run in parallel are shown at left.

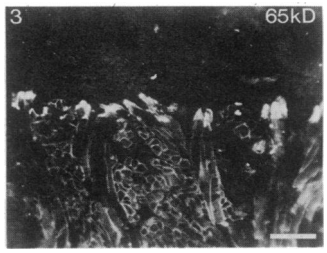

FIG. 3. Immunofluorescence staining of monkey tongue tissue with polyclonal antibodies to the 65-kDa polypeptide. Note that the pattern of staining is identical to that obtained with monoclonal antibody 4F11 (Fig. 1c). (Bar = 25 μm.)

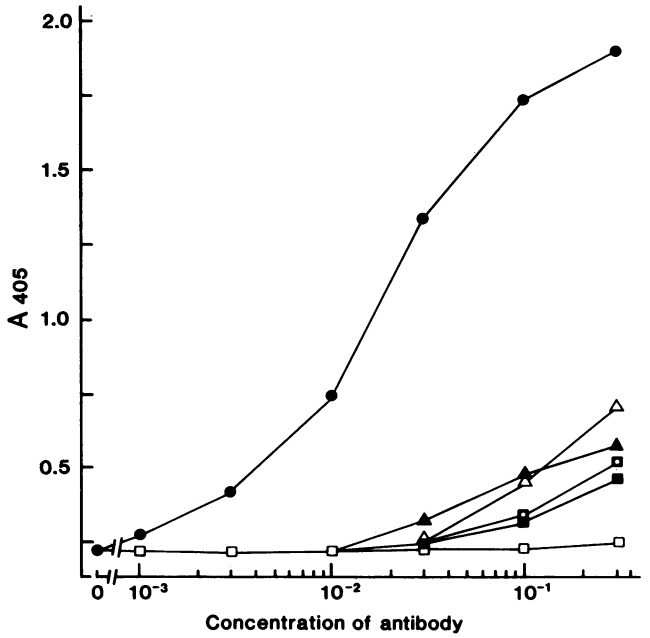

FIG. 4. Reactions of polyclonal antibodies to the 65-kDa polypeptide with various proteins in enzyme immunoassay. Bound antibodies were detected by alkaline phosphatase-conjugated goat anti-rabbit IgG and enzyme-catalyzed dye reaction. Wells were coated with 65-kDa polypeptide (●), human plasma fibronectin (▲), rat laminin (△), human laminin (□), mouse type IV collagen (■), and bovine serum albumin (□).

Identification of the Intact Polypeptide. Immunoblotting was used to determine the size of the tissue precursor of the 65-kDa polypeptide. For this purpose, a second generation of monoclonal antibodies were made against the denatured 65-kDa polypeptide. These antibodies stained basement membranes the same way as the original monoclonal antibodies generated against placental extracts (1B4 and 4F11) and the polyclonal antibodies against the native 65-kDa polypeptide. In immunoblotting, all seven of the antibodies detected an ≈80-kDa polypeptide in $NaDodSO_4$ extracts of placenta, but nothing was detected in extracts of kidney containing comparable amounts of protein (Fig. 5). Unreduced, the protein had an apparent molecular mass of about 70 kDa. The shift in electrophoretic mobility between the unreduced and reduced form of this protein resembled that observed with the 65-kDa pepsin fragment (Fig. 5). Monoclonal antibodies to laminin and type VI collagen were used as controls in this test and they detected high molecular weight laminin and collagen, respectively, in both tissue extracts. Unconditioned culture medium gave no staining (data not shown).

Expression of the 80-kDa Polypeptide in Cultured Cells and Developing Tissues. Culture media of human cell lines labeled with [^{35}S]methionine were subjected to immunoprecipitation with the monoclonal and affinity-purified polyclonal antibodies. A total of 28 human and rat cell lines were tested, including choriocarcinomas, rhabdomyosarcomas, schwannoma, a myoblast line, and other cells (see *Materials and Methods*). These cell lines were invariably negative in immunoprecipitations and in immunofluorescence of cell layers (data not shown). Control precipitations using antibodies to laminin, fibronectin, and complement component C3 (15) gave positive results. Similarly, laminin and fibronectin could be detected with monoclonal antibodies in immunofluorescence of cultured cells. Sections of two human choriocarcinoma tumors (BeWo and JAR) grown in nude mice were negative when stained with the monoclonal or polyclonal antibodies to the 65-kDa polypeptide but positive when stained with antibodies to laminin or fibronectin (data not shown).

To study the developmental expression of the 80-kDa polypeptide, we used the rabbit antibodies to examine pre- and postnatal stages of intercostal and hindlimb muscle development in the mouse, starting from day 15 of gestation when the first skeletal muscle basement membranes appear (30). We found that all prenatal stages, which include the appearance of primary, secondary, and tertiary myofibers, were negative for the 80-kDa polypeptide (Fig. 6*b*). Only in the first postnatal days were basement membranes significantly positive in intercostal muscles (Fig. 6*c*). Basement membranes in the sciatic nerve stained for the 80-kDa polypeptide only after the fifth postnatal day. Adult mouse skeletal muscle and sciatic nerve Schwann cell basement membranes were strongly stained by the antibodies (Fig. 6*d*).

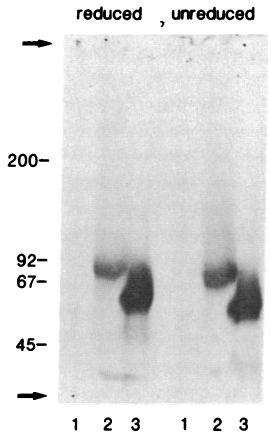

FIG. 5. Immunoblotting of human tissue extracts with monoclonal antibodies. Kidney (lanes 1) and placenta (lanes 2) were extracted with $NaDodSO_4$ as described in *Materials and Methods*, and extracts were electrophoresed together with purified 65-kDa fragment (lanes 3), before and after reduction, in a 2–16% gradient gel. The proteins were transferred electrophoretically to nitrocellulose and treated with monoclonal antibody and peroxidase-conjugated second antibody. Arrows indicate the top and bottom of the gel.

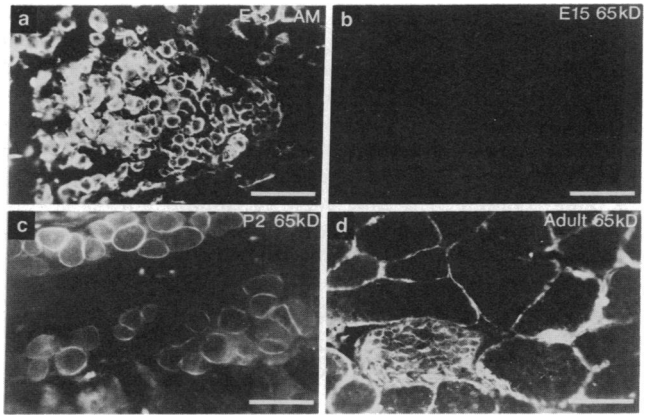

FIG. 6. Immunofluorescence staining of developing and adult mouse intercostal and hindlimb muscles. (*a* and *b*) Embryonic day 15. Anti-rat laminin staining (*a*) outlines basement membranes around developing myoblasts. Lack of staining with antibodies to the 65-kDa polypeptide indicates lack of merosin at this stage. (*c*) Postnatal day 2. Staining with antibodies to the 65-kDa polypeptide shows the appearance of merosin associated with the basement membranes of intercostal muscle cells. (*d*) Adult mouse hindlimb muscle. Distinct merosin immunofluorescence is seen in basement membranes of muscle cells and intramuscular nerve Schwann cells. (Bars = 25 μm.)

All the characteristics described above for this polypeptide establish it as a tissue-specific basement membrane protein. We have named this protein merosin (*meros*, Greek for to separate into compartments), since merosin compartmentalizes Schwann cells, muscle cells, and trophoblast cells from the interstitial matrix.

DISCUSSION

The present results identify a basement membrane-associated component, merosin, which has a restricted tissue distribution in basement membranes of Schwann cells, skeletal muscle, and trophoblast. Identification of merosin as a basement membrane-associated protein rests on the reactivity of several monoclonal antibodies with basement membranes from some but not all tissues, on the isolation of a unique 65-kDa polypeptide by affinity chromatography on some of these antibodies, and on the identification of an 80-kDa tissue protein that is present only in tissues positive in immunofluorescence. The identification was further confirmed by an immunohistological analysis of the reactivity of polyclonal antibodies prepared against this protein, merosin, and by its distinct immunological and developmental properties.

The pattern of tissue-specific basement membrane staining obtained with the monoclonal and polyclonal anti-merosin antibodies is unique and does not correspond to the distribution of any basement membrane-associated molecule known to us. Since the staining pattern of a polyclonal antiserum to extracellular macromolecules is generally thought to reflect the true distribution of the antigenic molecules, we assume that this is also the case for merosin. In early embryonic tissues, some extracellular protein antigens may be covered by glycosaminoglycans and have been exposed for immunodetection by digesting tissues with glycosaminoglycan hydrolases (31). In our study, however, failure of proteases and glycosaminoglycan hydrolases to uncover any merosin from unstained structures supports the observation of a restricted tissue distribution for this protein.

Monoclonal antibody INO (32) gives immunofluorescence staining of striated muscle and Schwann cell basement membranes that resembles our results. However, INO also stains other structures, including nuclei, the perineurium, and the glomerular capsule, suggesting that the antigen is different from ours. Other monoclonal antibodies (33) stain the periphery of skeletal muscle fibers, but the distribution of their staining in tissues such as smooth muscle, vessel walls, and nerves differs from that of merosin. Since the staining pattern of a monoclonal antibody such as INO may not always reflect the entire tissue distribution of the antigenic molecule (12, 16, 33), direct comparison to our results, which were confirmed immunohistochemically with a polyclonal antibody and by specific immunoblotting of tissue extracts, is not possible.

That merosin is a distinct protein is supported by the fact that the 65-kDa merosin fragment and its 80-kDa precursor were immunologically distinct from laminin, type IV collagen, and fibronectin. Since it was not possible to isolate merosin from tissue without proteolytic or denaturing treatments, we do not know whether the 80-kDa polypeptide represents intact merosin or whether it might be a subunit of a larger molecule. The difficulty of extracting merosin from tissue suggests that it is tightly bound in tissues.

The absence of merosin from a large number of human and rat cell lines tested suggests that the protein may be expressed by highly differentiated cells only. Results on the developmental appearance of merosin agree with the idea that the protein is expressed only in advanced stages of differentiation. Such differentiation may not take place in cultured cell lines. In any case, the developmental appearance of merosin in mouse muscles and nerves several days after the deposition of laminin and type IV collagen into basement membranes (30) indicates that merosin is not a basic structural component of these basement membranes. The late developmental appearance of merosin is unique among antigens of the basement membrane zone and, perhaps relates to the functional maturation of muscles and peripheral nerves indicated by the appearance of, for example, myoglobin (34) and myelin (35).

Present results establish a light microscopic localization of merosin to the basement membrane zone, and the ultrastructural localization of the protein relative to the various layers of the basement membrane zone remains to be determined. Regardless of the electron microscopic localization, however, the late appearance of merosin and its tissue specificity suggest that the protein plays a specific functional role associated with a high level of differentiation in tissues that express it. The identification of merosin may open new possibilities for studies on the development and functions of nerve, muscle, and placenta.

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