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Distribution and isolation of four laminin variants; tissue restricted distribution of heterotrimers assembled from five different subunits

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The distribution of subunits of the basement membrane proteins laminin and merosin in human and rabbit tissue was studied by immunofluorescence using monoclonal antibodies. The laminin A chain is present in epithelial, endothelial, and smooth muscle basement membranes. Merosin, as defined by its heavy chain M, is present in striated muscle and peripheral nerve. The A subunit colocalizes with at least two B subunits: B2 plus either B1 or the recently discovered B1 homologue S. The M subunit most often colocalizes with B1 and B2. Exceptions include the myotendinous junction, where M colocalizes with S, and the trophoblast basement membrane, where the M subunit colocalizes with S as well as B1. The presence of all five known subunits of the laminin family in placenta allowed isolation of their parent molecules in native form by the use of monoclonal antibodies in affinity chromatography. Four different heterotrimeric proteins could be identified: B1 chain–containing laminin (A-B1-B2), S chain–containing laminin (A-S-B2), B1-containing merosin (M-B1-B2), and S-containing merosin (M-S-B2). The data show that the proteins in the laminin family are heterotrimers composed of one heavy and two light chains; that most basement membranes contain predominantly one protein of the laminin family; and that laminin, as defined by the A subunit, has a much more restricted distribution than previously thought.

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Introduction

Laminin is a large glycoprotein and an integral component of basement membranes (for reviews see Martin and Timpl, 1987; Beck *et al.*, 1990; Yurchenco and Schittny, 1990). The most studied laminin comes from embryonic rodent tumor cells and is composed of three subunits: two distinct 200-kDa chains, designated as B1 and B2, and a 400-kDa A chain (Chung *et al.*, 1977, 1979; Timpl *et al.*, 1979). The B1 and B2 chains are homologous to each other and to the N-terminal two-thirds of the A chain (Sasaki *et al.*, 1987, 1988; Sasaki and Yamada, 1987; Durkin *et al.*, 1988). The laminin molecule has an asymmetric crosslike structure with three short arms and one long arm. The N-termini of the B1, B2, and A subunits separately form the three short arms. The stem of the long arm is composed of all three chains folded together in a coiled-coil structure. The C-terminal one-third of the A chain forms the large globular domain at the end of the long arm.

Several biological activities have been assigned to laminin. It promotes cell attachment, spreading, motility, and neurite outgrowth, as well as cell proliferation and differentiation. A number of cellular receptors for laminin have been identified. Laminin-binding integrins with different alpha and beta subunit compositions have been identified in various types of cells, including sarcoma cells, endothelial cells, platelets, and neuronal cells (Horwitz *et al.*, 1985; Gehlsen *et al.*, 1988, 1989; Ignatius and Reichardt, 1988; Sonnenberg *et al.*, 1988; Elices and Hemler, 1989; Kramer *et al.*, 1989; Languino *et al.*, 1989; Turner *et al.*, 1989; Kirchhofer *et al.*, 1990; Lotz *et al.*, 1990). It is not known which laminin subunit(s) these receptors recognize.

Laminin has previously been known as one distinct protein. However, recent findings have shown that it is a member of a family of proteins. There are at least two other related proteins, merosin and s-laminin in this family. Merosin was identified as a protein restricted to basement membranes of trophoblast, Schwann

cells, and striated muscle (Leivo and Engvall, 1988). Cloning and sequencing of the C-terminal portion of the merosin polypeptide, M, showed that it is structurally related to the laminin A chain (Ehrig *et al.,* 1990). Isolation of the native merosin protein revealed further similarities to laminin: the M polypeptide is similar in size to the laminin A chain, it is disulfide-crosslinked to light chains similar or identical to B1 and B2, and has a crosslike structure similar to that of laminin. S-laminin (synaptic laminin), the other recently characterized laminin-related polypeptide, is a B1 homologue (Hunter *et al.,* 1989). Whether the S chain is associated with other polypeptides in a laminin-like protein has not been determined previously. Other laminin variants that may be additional members of the laminin family have been described but not yet structurally characterized (Davis *et al.,* 1985; Lander *et al.,* 1985; Aratani and Kitagawa, 1988; Edgar *et al.,* 1988).

Immunohistochemical studies using polyclonal antibodies led to the belief that laminin is present in all basement membranes throughout development. However, measurements of mRNA levels for the different laminin subunits in tissues have shown that, whereas the B subunits are expressed at high levels in many tissues, the A subunit is expressed at very low levels or not at all in some tissues (Laurie *et al.,* 1989; Olsen *et al.,* 1989). In part this could be because of the low turnover of basement membranes. Also, because the synthesis of the A chain seems to be the rate-limiting step in the synthesis and secretion of laminin (Peters *et al.,* 1985), there may be an excess of the B chains synthesized. However, in some developing tissues, A-chain expression can be detected only transiently (Ekblom *et al.,* 1990), strongly suggesting that laminin is not present in all basement membranes.

The finding that merosin is a muscle- and nerve-specific laminin-like basement membrane protein raised the question of whether these basement membranes might contain merosin instead of laminin. To explore this possibility and to analyze the B-chain associations of the A and M polypeptides, we have developed chain-specific antibodies to localize the various subunits in tissues, to isolate the various laminin-like proteins, and to analyze their subunit composition.

We show here and in a separate paper (Sanes *et al.,* 1990) that the A and M chains indeed have mutually exclusive distributions; most basement membranes could be stained with either anti-M or anti-A. Furthermore, the M chain seems to associate more often with the B1 than with the S subunit, whereas the A chain is frequently associated with S. The associations of one heavy chain (A or M) with a B1 or S subunit and a B2 subunit were confirmed by analysis of the isolated molecules.

Results

Characterization of monoclonal antibodies to the laminin A chain

Monoclonal antibodies were prepared against proteins that were isolated from a placental pepsin extract by affinity chromatography on a monoclonal anti-B1 chain antibody column (Wewer *et al.,* 1983; Engvall *et al.,* 1986). Ten antibodies that reacted with the pepsin fragments in immunoblotting were further characterized by immunofluorescence on sections of placenta and fetal membranes. Two potential M-chain antibodies were identified in this test by their staining of the trophoblast basement membrane but not the vascular basement membrane in placental sections (Leivo and Engvall, 1988). The other 8 antibodies stained both the trophoblast and the vascular basement membranes in placenta. These antibodies were assumed to be against the B1, B2, or A chain.

To establish which of the eight antibodies were B-chain specific, they were tested against purified merosin in immunoblotting. Six antibodies stained one or the other of the B chains

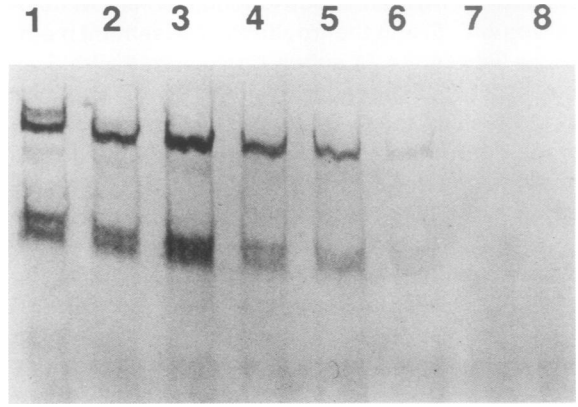

Figure 1. **Immunoprecipitation from conditioned medium of JAR choriocarcinoma cells incubated with (^{35}S) methionine.** The antibodies used were: 2E8 anti-B2 (lane 1), 4E10 anti-B1 (lane 2), 4C7 anti-A (lane 3), 2C4 anti-B (lane 4), 1F5 and 11D5 anti-A (lanes 5 and 6), 1F9 anti-M (lane 7), and control antibody 3E1 (anti-integrin beta 4, lane 8). The 7% acrylamide gel was run under reducing conditions.

Table 1. Summary of characteristics of monoclonal antibodies

Antibody	Ig class	Subunit specificity	Species reactivity	Reference
1F5	IgG1	A	human	This paper
11D5	IgG1	A	human	This paper
4C7	IgG2a	A	human rabbit	Engvall *et al.*, 1986
5H2	IgG1	M	human rabbit	Leivo and Engvall, 1988
2G9	IgG1	M	human rabbit	Leivo and Engvall, 1988
4E10	IgG1	B1	human rabbit	Wewer *et al.*, 1983; Engvall *et al.*, 1986
3E5	IgG1	B1	human	Engvall *et al.*, 1986; Gehlsen *et al.*, 1989
2E8	IgG2a	B2	human rat	Engvall *et al.*, 1986
C1	IgG1	S	human rabbit rat	Hunter *et al.*, 1989; Sanes *et al.*, 1990
C4	IgG1	S	human rabbit rat guinea pig	Hunter *et al.*, 1989 Sanes *et al.*, 1990

in merosin. The remaining two antibodies, 1F5 and 11D5, did not stain the B chains or the M chain and were considered candidates for being A-chain antibodies.

JAR choriocarcinoma cells synthesize and secrete laminin (Peters *et al.*, 1985). The 400-kDa chain of the JAR-cell laminin is the human equivalent of the mouse Engelbreth-Holm-Swarm tumor (EHS) A chain, as shown by extensive homologies of the cDNA sequences for the two polypeptides (T. Haaparanta, J. Uitto, E. Ruoslahti, and E. Engvall, unpublished observations). The presumed A-chain-specific monoclonal antibodies, 1F5 and 11D5, precipitated laminin from JAR-cell culture medium, as did known B-chain antibodies, whereas merosin antibodies did not (Figure 1). Because JAR cells make only two B subunits (B1 and B2) and one A chain, and because 1F5 and 11D5 were not against B1 or B2, they have to be A-chain specific. These and other antibodies used here to localize laminin and merosin subunits are listed in Table 1.

Laminin A chain and merosin M chain in different basement membranes

Earlier results (Leivo and Engvall, 1988) have shown that merosin, as defined by its M chain, is abundant in the basement membrane of adult skeletal muscle fibers. It was therefore of interest to study the distribution of the A chain in this location. Staining with the A chain–specific antibodies in immunofluorescence revealed a striking lack of staining in the muscle fiber basement membrane in tongue (Figure 2A). The antibodies strongly stained blood vessels in muscle and dermis as well as the epidermal basement membrane. Anti–A chain antibodies 1F5 and 11D5, as well as the previously characterized antibody 4C7, gave the same staining pattern in all human tissues. Antibody 4C7 crossreacts with rabbit basement membranes, allowing the use of the more readily available rabbit tissues for some experiments. Based on the results in Figure 2, A and B, it appears that the basement membrane of the mature muscle fiber contains predominantly merosin (the M chain associated with B chains) and little or no laminin (the A chain associated with B chains).

The presence of laminin and merosin in basement membranes other than skeletal muscle was investigated (Figure 2, C–J). In the heart, laminin A chain was absent from the muscle but present in blood vessels, similar to what was found in skeletal muscle (Figure 2C). The A chain was present, however, in the smooth muscle of human umbilical cord, monkey colon, and rabbit stomach and bladder, whereas the M chain was not detected in these tissues (Figure 2, E and

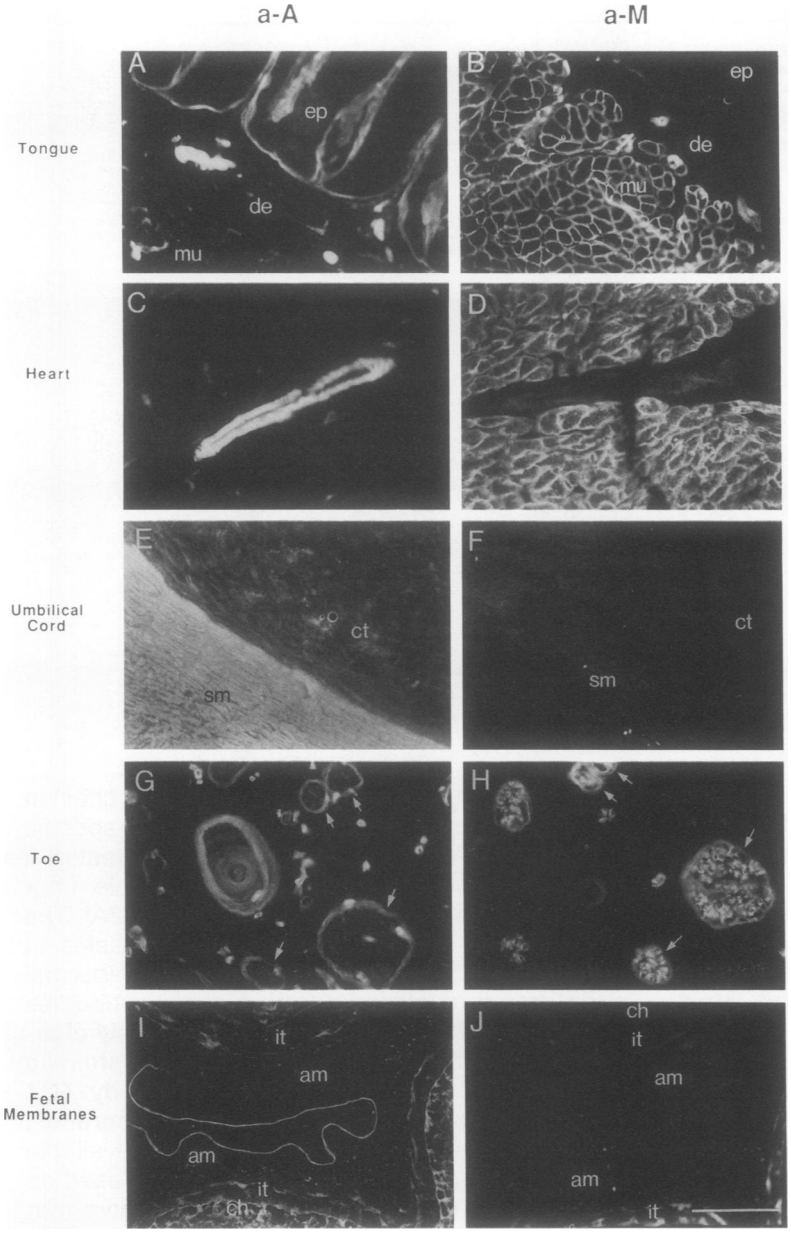

Figure 2. **Indirect immunofluorescence on tissue sections using monoclonal antibodies to the laminin A chain (antibody 4C7; left panels) or the merosin M chain (antibody 5H2; right panels).** (A and B) Adult rabbit tongue. ep, epidermis; de, dermis; mu, muscle. (C and D) Adult rabbit heart. (E and F) Human umbilical cord. sm, smooth muscle; ct, connective tissue. (G and H) Human toe tissue from 1-1/2-y-old infant. Arrows point to four different peripheral nerves. (I and J) Human fetal membranes. am, amnion; ch, chorion; it, intermediate trophoblast. Bar = 50 μm.

F; Leivo and Engvall, 1988). In peripheral nerve, A-chain staining was abundant in the perineurium, whereas the M chain was predominantly found in the Schwann cell basement membrane (Figure 2, G and H). In fetal membranes, the A chain was detected in all epithelial basement membranes of the amnion and chorion. The M chain was found only in the layer of the intermediate trophoblast cells (Figure 2, I and J). Thus, most basement membranes contain either the A chain or the M chain, but rarely both.

Laminin A chain and merosin M chain may associate with either the B1 chain or the S chain

The distribution of the B1 and B2 chains is widespread, but the distribution of the S chain is more restricted (Hunter *et al.,* 1989). The S chain is limited to synaptic sites in muscle, to the perineurium in peripheral nerve, and to certain blood vessels and the glomeruli in the kidney. These are all basement membranes that contain the A chain but not the M chain (Figure 2; Leivo and Engvall, 1988; Sanes *et al.,* 1990).

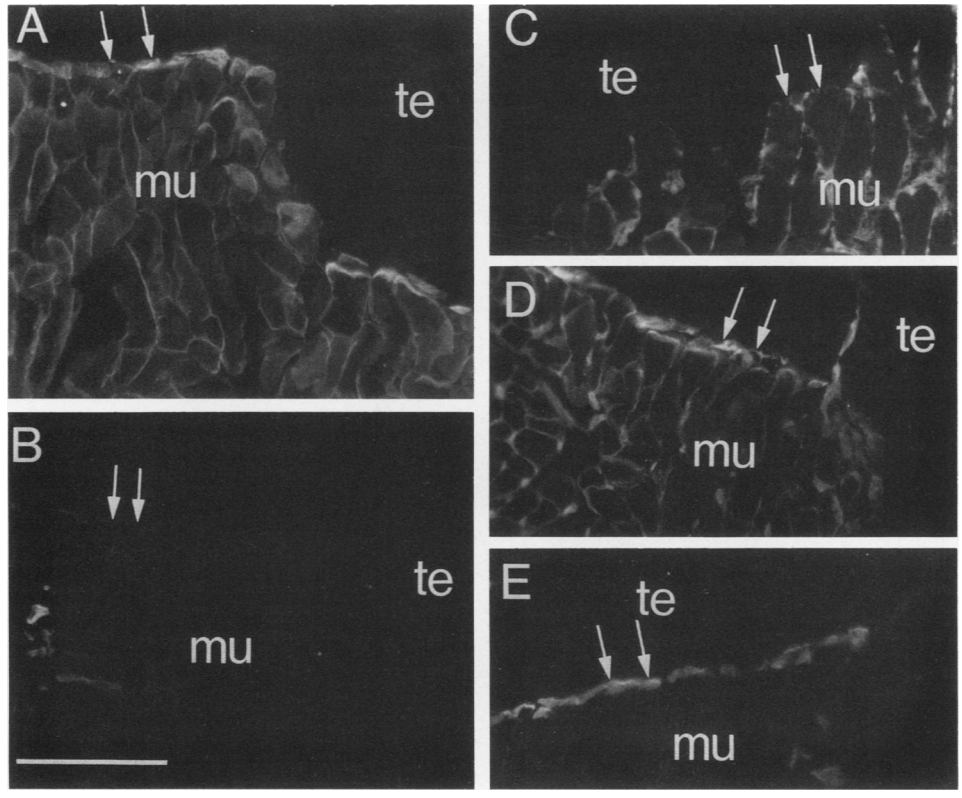

Figure 3. **Laminin and merosin in the myotendinous junction.** Sections of toe muscle from 1-1/2-y-old infant were stained with monoclonal antibodies in indirect immunofluorescence. (A) anti-M (5H2); (B) anti-A (4C7); (C) anti-B1 (4E10); (D) anti-B2 (2E8); (E) anti-S (C4). mu, muscle; te, tendon. Double arrows indicate the myotendinous junction. Bar = 50 μm.

These results suggested that laminin may have the subunit composition A-B1-B2 or A-S-B2, whereas merosin would only occur as the trimer M-B1-B2. However, we noticed one basement membrane that contained M but appeared to lack B1, that of the myotendinous junction (Figure 3). As previously noted (Leivo and Engvall, 1988), muscle attachment sites contain an apparent accumulation of merosin. The same increased staining pattern was seen with the B2 antibody, but not with the B1 or with the A-chain antibodies (Figure 3). However, antibodies against S-laminin intensely and selectively stained the muscle attachment sites. These results suggest that molecules with the composition M-S-B2 are responsible for the staining pattern at the myotendinous junction.

Isolation of S-merosin from placenta

Placenta is a rich source for both laminin and merosin (Wewer *et al.,* 1983; Dixit, 1985; Ohno *et al.,* 1983). The placenta is also rich in vasculature and might contain S-laminin, which is found in many blood vessels. We therefore stained placental sections with antibodies to the different laminin and merosin subunits (Figure 4). The A, B1, and B2 subunits are present in both the trophoblast basement membrane and the basement membrane of the fetal capillaries, whereas the M subunit is present only in the trophoblast basement membrane. Surprisingly, the S-subunit antibodies stained predominantly the trophoblast basement membrane.

To determine whether placenta contains merosin with the subunit composition M-S-B2, in addition to merosin with the subunit composition M-B1-B2 (Ehrig *et al.,* 1990), we isolated small amounts of merosin from EDTA extracts of placenta by using an M-chain-specific antibody. This preparation of merosin was tested for the presence of B1, B2, and S chains by ELISA (not shown) and by immunoblotting (Figure 5). Antibodies against B1, B2, and S all reacted predominantly with ~200-kDa polypeptides in the merosin preparation. Fainter bands of higher molecular weight may represent crosslinked B chains. The anti-S antibody stained additional bands below the 200-kDa region. Because the number and position of these extra bands varied between preparations, it is possible that they represent degradation products. Antibodies against the 300-kDa and the 80-kDa portions of the M chain reacted with the

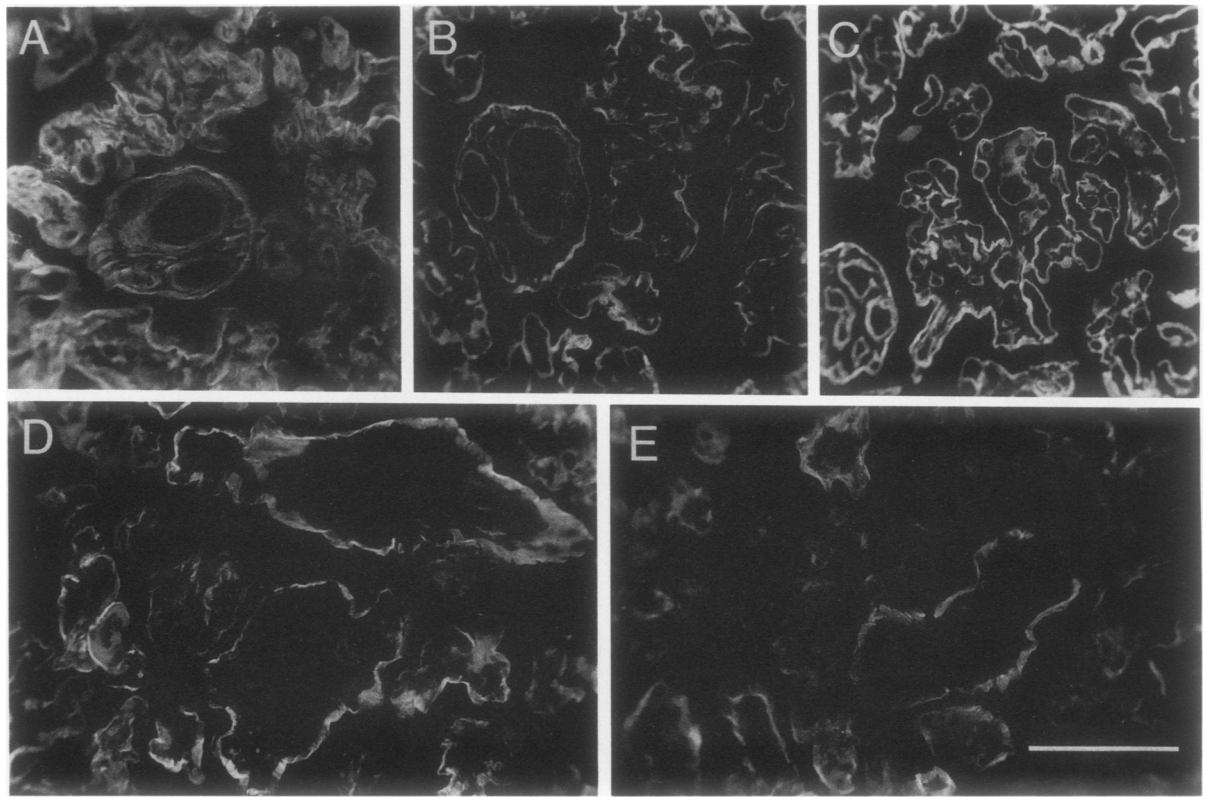

Figure 4. **Laminin and merosin subunits in placenta.** Sections of term placental villi were stained with monoclonal antibodies in indirect immunofluorescence. (A) anti-A (4C7); (B) anti-B1 (4E10); (C) anti-B2 (2E8). (D) anti-M (5H2); (E) anti-S (C4). Bar = 50 μm.

300-kDa (not shown) and the 80-kDa (Figure 5) polypeptides, respectively. Thus, the merosin prepared this way contained all three known B chains, presumably in pairs of either B1-B2 or S-B2.

Because isolation of intact merosin and laminin by antibody affinity chromatography results in very poor yields (our unpublished observation), we isolated truncated merosin and laminin from pepsin digests of placenta. Four types of samples were prepared; three were obtained by affinity chromatography on anti-B1, anti-M, and anti-A antibodies, respectively. The fourth preparation was prepared by affinity chromatography on the anti-M antibody of an extract that had been depleted of B1-reactive material by repeated passage through the anti-B1 column. The four samples were then tested for the presence of the different laminin and merosin subunits by binding of subunit-specific antibodies in ELISA (Figure 6). The laminin subunit S was not present in the sample that was isolated on an anti-B1 column, supporting the hypothesis that S can substitute for B1 but is not present in the same molecules as B1. The materials isolated with either the A- or the M-specific antibodies contained low but significant levels of the S subunit. The highest level of S subunit was present in the merosin preparation isolated from a B1 subunit–depleted digest. The B1 subunit was present in each of the preparations that had not been specifically depleted of B1, whereas the B2 subunit was present at similar levels in all four preparations. The specificity of the isolation procedure was established by the absence of the M subunit in the anti-A isolated material and the low amount of the A subunit in the anti-M isolated preparation.

Discussion

It has only recently become apparent that basement membranes contain more than one laminin-like protein. This went unnoticed for quite some time because laminin contains several subunits and the recently discovered laminin-like proteins, merosin (Leivo and Engvall, 1988; Ehrig *et al.,* 1990) and S-laminin (Hunter

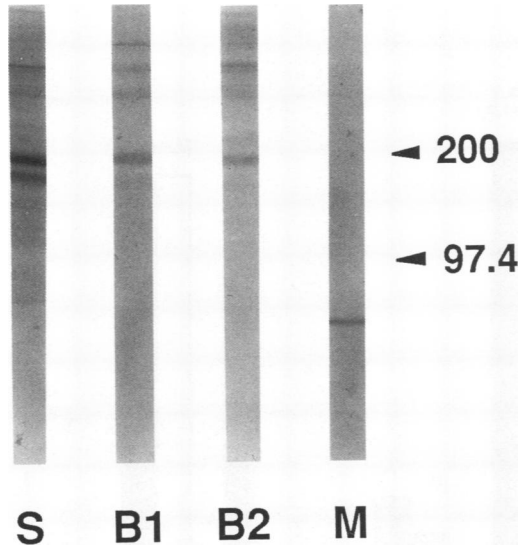

Figure 5. **Immunoblotting of isolated intact merosin with antibodies against S (C4), B1 (3E5), B2 (2E8), and the 80-kDa fragment of the merosin M chain (5H2).**

et al., 1989), share with laminin some of the subunits. The data presented in this paper show that the various polypeptides of the laminin-like proteins exhibit distinct and, at times, mutually exclusive tissue distributions and that all of the four expected heterotrimeric permutations (containing 1 heavy chain, 1 B1-like chain, and 1 B2 chain) of the five known subunits can be isolated from tissues.

Our results reveal an interesting reciprocity in the tissue distributions of the alternative subunits in the laminin-like heterotrimers. The M chain of merosin is homologous to the laminin A chain, and the two have similar sizes. In the heterotrimer either one of these subunits is associated with two B chains to form either the A chain–containing laminin or the M chain–containing merosin. Most of the basement membranes we studied contained either the A chain or the M chain but not both. One exception is the trophoblast basement membrane, which contains both of these subunits. This basement membrane may be contributed to by several cell types such as the trophoblast, the syncytiotrophoblast, and the intermediate trophoblast cells. Another exception is synaptic basal lamina in muscle, which may also receive contributions from several cell types and contains both A and M subunits (Sanes *et al.,* 1990). The reciprocal expression of the A and M chains in many tissues suggests that the heterotrimers characterized by the presence of these subunits are funtional alternates for one another in the basement membrane. The two proteins therefore are likely to have different functions, despite the apparent similarity of their activities.

The S chain is most closely homologous to the B1 chain and, as was the case for the A and M chains, the tissue distributions of the S and B1 chains were reciprocal; most basement membranes contain predominantly one or the other. Staining for the B2 chain was found in all basement membranes examined, suggesting that homologues of this subunit may not exist. Alternatively, our antibody may recognize a conserved region of other B2-like subunits.

The tissue distribution of the S chain corresponded to that of the A chain rather than the M chain in most tissues, suggesting that the S chain is preferentially included in heterotrimers with the A chain. One exception, however, the myotendinous junction, suggested that heterotrimers containing the S chain and the M chain also exist.

Direct analysis of isolated heterotrimers from placenta confirmed the predictions made about the subunit compositions from the staining results. A total of four heterotrimers were identified and their subunit compositions were shown to correspond to the expected permutations A-B1-B2 (which is the classical laminin), M-B1-B2 (which is the original merosin), and the S chain-containing forms of each, A-S-B2 and M-S-B2. The availability of these laminin-like proteins and antibodies against them will now allow analysis of their functional properties. A large number of integrins and other molecules have been described that are thought to mediate cell adhesion to laminin. It will be interesting to see whether some of them might be specific for individual members of the laminin family and what functional consequences such specificities might have for the cells.

Methods

Isolation of laminin and laminin-like molecules

Intact merosin was isolated from placenta after EDTA extraction (Paulson and Saladin, 1989) either by gel filtration on Sepharose 6B and ion exchange chromatography (Ehrig *et al.,* 1990) or by monoclonal antibody affinity chromatography on antibody 5H2 (Leivo and Engvall, 1988), an M chain–specific antibody. Large pepsin fragments of laminin and merosin were prepared from pepsin digests of term placenta by monoclonal antibody affinity chromatography (Wewer *et al.,* 1983; Engvall *et al.,* 1986) using specific antibodies.

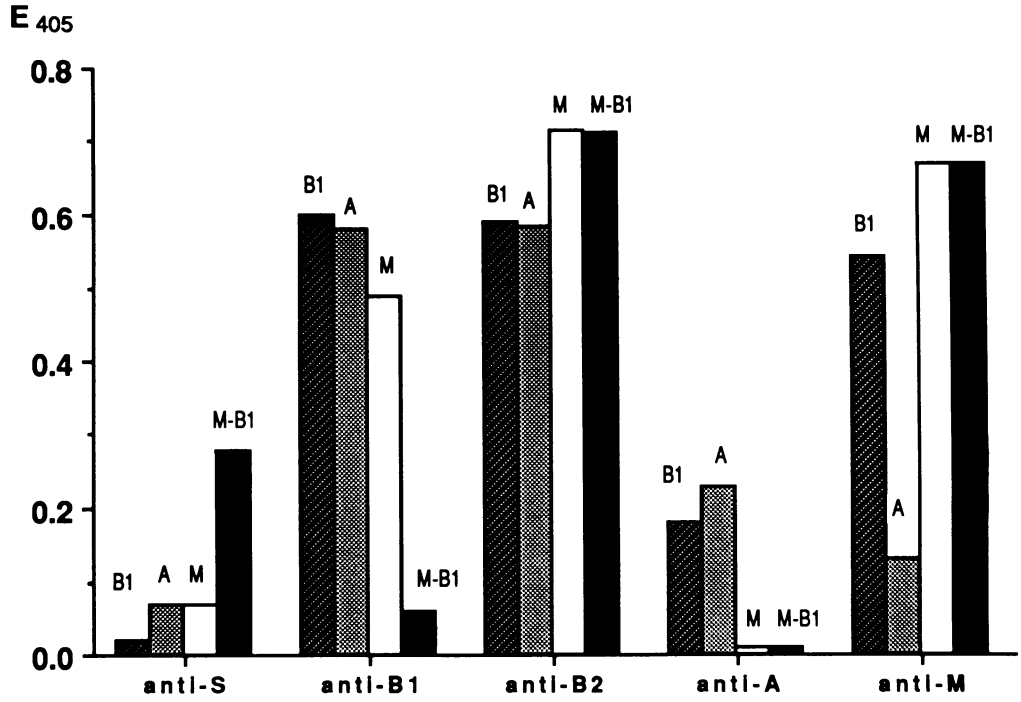

Figure 6. **Laminin and merosin subunits in affinity-purified preparations as measured by ELISA.** Protein was isolated from a pepsin digest of placenta by affinity chromatography on 4E10 anti-B1 Sepharose (B1, □), 4C7 anti-A sepharose (A, □), and 5H2 anti-M Sepharose (M, □). Protein was also isolated from a digest, which had been depleted of anti-B1 reactive material, on 5H2 anti-M Sepharose (M-B1, ■). Microtiter wells were coated with ~1 μg/ml of each preparation. The relative amounts of the different subunits in the preparations were determined after incubation with antibodies C4 (anti-S), 4E10 (anti-B1), 2E8 (anti-B2), 11D5 (anti-A), and 2G9 (anti-M). Bound antibody was determined after incubation with alkaline phosphatase labeled anti-mouse IgG and measurement of bound enzyme activity (E 405).

Antibodies

Several monoclonal antibodies against purified pepsin fragments of laminin have been described previously (Engvall *et al.*, 1986). Three of these antibodies have been shown to recognize the B1 and B2 chains of laminin: antibody 2E8 crossreacts with rat L2 laminin and binds to the smallest of the rat laminin subunits, the B2, in immunoblotting. Two other antibodies, 3E5 and 4E10, which were initially thought to be A-chain specific (Engvall *et al.*, 1986) have since been shown to be specific for the B1 chain (Gehlsen *et al.*, 1989). Another antibody, 4C7, binds to the globular domain at the end of the long arm (Engvall *et al.*, 1986). This domain is the C-terminal portion of the A chain and the 4C7 antibody could therefore be against the A chain. However, previously it has not been possible to assign a chain specificity to this antibody because it does not crossreact with rodent laminin and does not work in immunoblotting. Monoclonal antibodies 5H2 and 2G9 against the C-terminal portion of merosin have been described (Leivo and Engvall, 1988). Two monoclonal antibodies against S-laminin that crossreact with human laminin, C1 and C4, have been described (Hunter *et al.*, 1989).

A new set of monoclonal antibodies was produced here for the purpose of obtaining A chain–specific antibodies. Balb/c mice were immunized with the pepsin fragments isolated from placenta by an anti-B1 subunit–specific antibody, and the immune spleen cells were fused with the myeloma cell line ag8.653 using polyethylene glycol (Hessle *et al.*, 1984). The characteristics of the monoclonal antibodies used here are summarized in Table 1.

Immunoassays

Indirect immunofluorescence was performed essentially as described (Leivo and Engvall, 1988). Cryostat sections (5-μm) of various tissues were air dried and fixed in acetone before incubation with monoclonal antibodies and fluorescein-conjugated second antibodies (Sigma, St. Louis, MO).

Immunoblotting using monoclonal antibodies has been described (Leivo and Engvall, 1988).

Enzyme-linked immunosorbent assay (ELISA) was performed as described (Engvall, 1980) by incubating dilutions of antibodies in microtiter wells coated with 1 μg/ml of protein. Bound antibodies were detected by alkaline phosphatase–labeled anti-immunoglobulin (BioRad Laboratories, Richmond, CA).

Immunoprecipitations were performed as described (Hessle and Engvall, 1984) from conditioned medium of metabolically labeled JAR choriocarcinoma cells.

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