A Synaptic Localization Domain in the Synaptic Cleft Protein Laminin β2 (s-Laminin)

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The basal lamina that ensheaths skeletal muscle fibers traverses the synaptic cleft at the neuromuscular junction. Synaptic and extrasynaptic portions of the basal lamina contain different laminin β chains: β2 (or s) at synapses and β1 (or B1) extrasynaptically. Laminin β2 is also confined to synapselike patches on myotube surfaces in vitro, whereas β1 is present throughout the extracellular matrix. This differential localization of laminin β chains was analyzed by expression of chimeric β1-β2 molecules in cultured mouse myotubes. A 16–amino acid carboxyl-terminal sequence in β2 was necessary for synaptic localization, and an amino-terminal domain in β1 promoted association with extracellular fibrils. The synaptic targeting sequence of β2 contains a site previously shown to be adhesive for motor neurons.

The postsynaptic apparatus of the skeletal neuromuscular junction is assembled from numerous extracellular, membrane, and cytoskeletal components (*1*). To date, most studies of the assembly of the postsynaptic apparatus have focused on a transmembrane protein, the nicotinic acetylcholine receptor (AChR), which is present at ≥10,000 copies per square micrometer in the postsynaptic membrane but at ≤10 copies per square micrometer in the extrasynaptic membrane (*2*). In contrast, little is known about the mechanism by which the components of the synaptic cleft are localized. We studied the localization of one such component, laminin β2 (s-laminin) (*3*, *4*).

The basal lamina that ensheaths each muscle fiber passes between the pre- and postsynaptic membranes at the neuromuscular junction. Laminin β2 is concentrated in the synaptic portion of this basal lamina. Adjoining extrasynaptic portions of the basal lamina contain little β2 but are rich in its homolog, laminin β1 (or B1) (*4*, *5*). Both β1 and β2 form heterotrimers with α and γ chains (*6*, *7*) (Fig. 1A), but they mediate distinct functions: β2 arrests the growth of axons promoted by β1-containing laminin trimers (*8*), and neuromuscular junctions are aberrant in mutant mice that lack β2 (*9*). The restriction of β2 to the synaptic cleft and of β1 to the extrasynaptic areas is therefore likely to be important for normal synaptic development.

In vivo, ingrowing motor axons induce the formation of postsynaptic specializations (*1*). In vitro, however, muscle cells cultured in the absence of nerves can assemble elaborate "hot spots" that include high-density clusters of AChRs (*2*, *10*) and several components of the postsynaptic cytoskeleton and synaptic cleft (*11*, *12*), including laminin β2. We were therefore able to use aneural cultures of a mouse muscle cell line, C2, to investigate the mechanism by which the laminin β1 and β2 chains become differentially distributed. C2 cells

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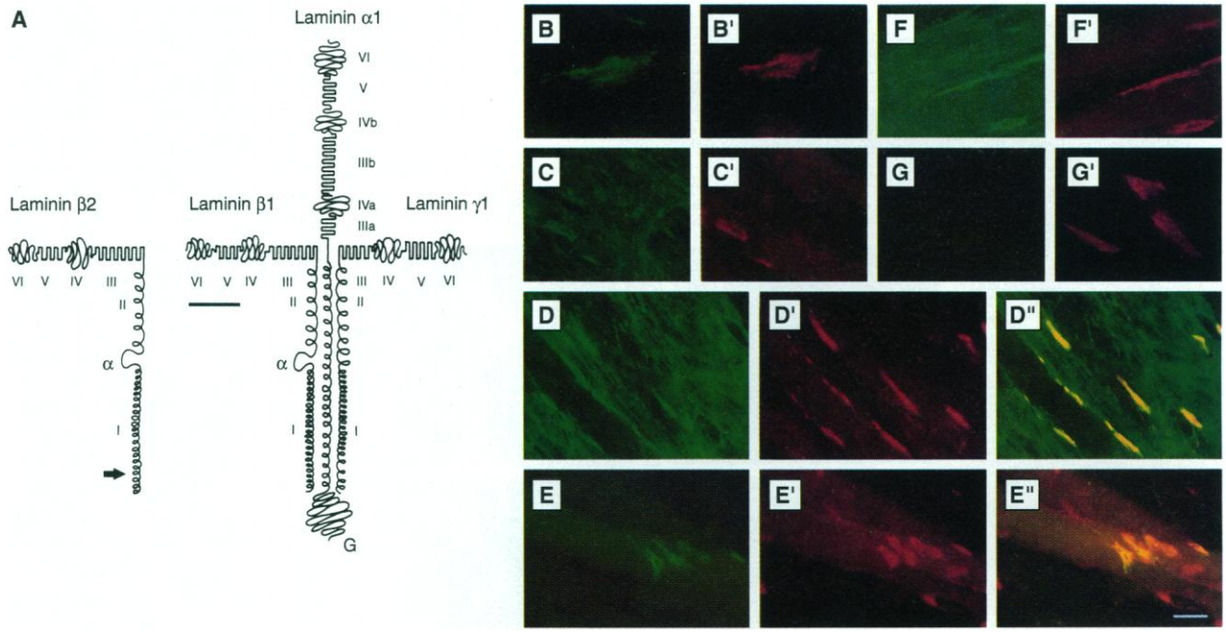

Fig. 1. Expression of the laminin β1 and β2 chains in murine C2 myotubes. (**A**) Structure of laminin. Laminin-1 is a cruciform trimer of three related chains, α1, β1, and γ1. The β2 chain is a homolog of β1, and substitutes for β1 to form alternative trimers (*5*–*7*). Roman numerals indicate structural domains deduced from the predicted secondary structure. G is the globular domain in α1. Synaptic- and matrix-localizing sequences defined in this study are indicated by an arrow and a bar, respectively. (**B** to **G**) Micrographs of C2 myotubes doubly stained with fluorescein-tagged antibodies to laminins (B) to (G) plus rhodamine-labeled α-bungarotoxin (B′) to (G′). The antibodies were specific for β2 (B), β1 (C), laminin-1 (D), or rat (but not mouse) β2 (E) to (G). The toxin labels AChRs and thus marks AChR-rich hot spots on the myotube surface. In (B) to (E), unpermeabilized cells were stained so that only extracellular antigen is visualized. In untransfected cells, β2 is confined to hot spots while β1 is diffusely distributed in the extracellular matrix [(B) and (C)]. In cells transfected with a rat β2 expression vector, the exogenous β2 is confined to hot spots, and the distribution of other laminin chains is undisturbed [(D) and (F)]. (D″) and (E″) are double exposures, in which regions of overlap between AChRs and β2 appear yellow. In (F), the cells were transfected with rat β2, stained with α-bungarotoxin, then fixed and permeabilized before being stained with antibody to rat β2. Exogenous β2 is diffusely distributed in the myotube cytoplasm, suggesting that posttranslational targeting rather than local synthesis leads to association with hot spots. In (G), the monoclonal antibodies to rat β2 used in (E) and (F) did not crossreact with endogenous mouse β2, as shown by their inability to stain hot spots in untransfected myotubes. Scale bar, 30 μm.

proliferate in rich medium, but fuse to form myotubes after withdrawal of serum (*13*). Soon after fusion, AChR-rich hot spots, detectable by staining with rhodamine–α-bungarotoxin, form on the myotube surface. Laminin β2 was colocalized with AChRs at these hot spots (*14*) (Fig. 1B). In contrast, laminin β1 (*15*) was widely distributed on the myotube surface (*16*) and in extracellular fibrils between myotubes (Fig. 1C).

Preferential synthesis of β2 near synaptic sites may contribute to its localization in vivo (*17*). To determine whether local synthesis underlies targeting in vitro, we transfected C2 myoblasts with a vector in which constitutively active regulatory elements from the Rous sarcoma virus directed expression of a full-length rat laminin β2 complementary DNA (cDNA) (*7, 18*). The vector also contained a gene that confers resistance to the antibiotic G418, allowing us to use drug selection to eliminate the untransfected cells (*18*). The transfectants were then stained with monoclonal antibodies to rat β2 that show no detectable cross-reaction with mouse β2 (*14*). When cells were fixed and permeabilized before staining, β2 immunoreactivity was present throughout the myotubes (Fig. 1F), suggesting that β2 synthesis was not restricted to regions near hot spots. However, extracellular β2 immunoreactivity was almost entirely confined to hot spots (Fig. 1E). The distribution of the other laminin chains was unperturbed (Fig. 1D), and untransfected cells were not stained by the monoclonal antibodies to rat β2, whether or not they were permeabilized (Fig. 1G). Thus, even when β2 protein was uniformly distributed within myotubes, extracellular β2 was restricted to hot spots.

These results suggest that differences in the primary structures of the laminin β1 and β2 chains are responsible for their different localizations. To identify the relevant structural differences, we prepared chimeric molecules in which portions of β2 were replaced by the corresponding regions of β1. Because β1 and β2 are homologous along their entire lengths and are virtually identical in domain structures (*5*), it was possible to construct chimeras without altering the length or predicted secondary structure of the proteins (*19*). All chimeras were characterized by transfection into fibroblasts, which do not synthesize detectable amounts of any endogenous laminin chain. Chimeric cDNAs generated full-length proteins that were detectable both by immunoblot analysis (*20*) and by immunofluorescence staining (*14*). The chimeras were then introduced into C2 cells, and stable transfectants were selected, fused to form myotubes, and stained with antibodies to rat laminin β2 (*21*). Both chimeras 1 and 2, in which domains IV and III, respectively, of β2 were replaced by β1 sequences (Fig. 2A), accumulated at hot spots in a fashion indistinguishable from that of full-length β2. Similar results were obtained with chimera 3, in which domains III and IV of β2 were both replaced by the corresponding region of β1 (Fig. 2, B and C). Chimeras 4 (Fig. 2, D and E) and 5, in which domains V and VI or domains III to VI, respectively, of β2 were replaced by β1 sequences, were also restricted to hot spots, although the hot spots were less strongly stained. In contrast, replacement of domains I and II of β2 with the corresponding β1 domains resulted in a protein (chimera 6) that was secreted and formed aggregates within the extracellular matrix but that was not associated with hot spots (Fig. 2, F and G). Thus, a site important for localization of β2 to hot spots is associated with domains I or II. This region was then subdivided in chimeras 7 and 8 (Fig. 2A). Restoring β2 sequence to the COOH-terminal 9% of chimera 6 generated chimera 7, which associated with hot spots. In contrast, replacement of the COOH-terminal 9% of β2 with the corresponding segment of β1 (chimera 8) was sufficient to prevent association of the chimera with hot spots. Thus, a synaptic localization domain is present in the COOH-terminal 9% of laminin β2.

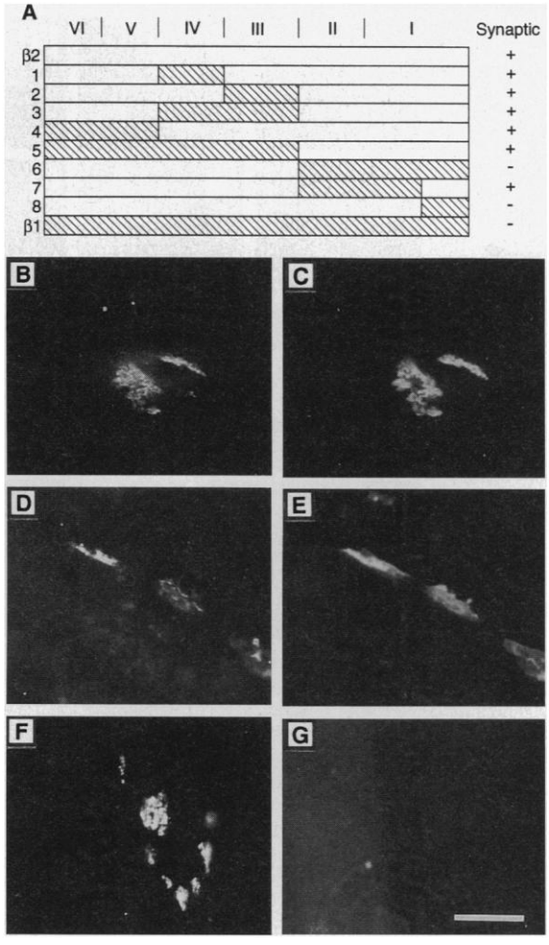

Fig. 2. Structure of β1-β2 chimeras and distribution of chimeric proteins. (**A**) Construction of chimeras 1 to 8. β1-derived portions are hatched and β2-derived sequences are open. Roman numerals indicate structural domains (Fig. 1A). Symbol at right of each construct indicates the distribution of the chimeric protein in transfected C2 myotubes: (+), confined to hot spots; (−), distributed in the extracellular matrix. (**B** to **G**) Transfected myotubes doubly labeled with monoclonal antibodies to rat β2 (B, D, and F) and rhodamine–α-bungarotoxin (C, E, and G). (B) chimera 3; (D) chimera 4; and (F) chimera 6. Antibodies were chosen to recognize β2 domains present in the chimeras (*21*). Cells were stained without permeabilization, so only extracellular antigen is visualized. The field shown in (F) and (G) contains a chimera-rich aggregate, but no AChR cluster. Scale bar, 30 μm.

To define the localization site further, we introduced small stretches of β2 sequence into the β1-derived portion of chimera 8, which was not localized to hot spots (Fig. 3A). Regions selected were those in which the β1 and β2 sequences were most divergent (*22*). Of the four chimeras in this set, chimera 9 was not secreted (*23*), chimeras 10 (Fig. 3, B and C) and 12 were secreted but not localized to AChR clusters, and only chimera 11 was localized to AChR clusters (Fig. 3, D and E). Chimeras 10 and 11 both formed heterotrimers with other laminin chains, suggesting that neither localization to nor exclusion from clusters reflected interference with chain assembly (*24*). Thus, chimera 11 defined a synaptic localization sequence in a 16–amino acid stretch within domain I of laminin β2.

Although chimeras with β1 sequence in the synaptic localization domain (chimeras 6, 8, 10, and 12) were associated with extracellular fibrils, they were not distributed identically to endogenous β1. Whereas β1 was diffusely distributed throughout the extracellular matrix (Fig.

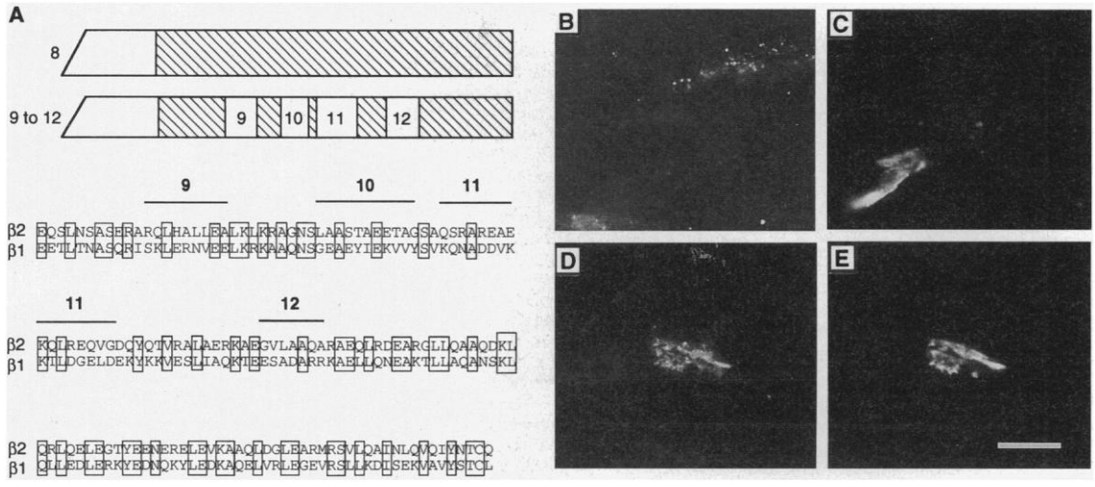

Fig. 3. Identification of a synaptic localization sequence in β2. (**A**) Comparison of β1 and β2 sequences in the COOH-terminal region defined by chimera 8. In regions where the two sequences are most dissimilar, 7– to 16–amino acid stretches of β1 were replaced by corresponding β2 sequence to generate chimeras 9 to 12. Of these, only chimera 11 was selectively associated with hot spots. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B** to **E**) Myotubes transfected with chimeras 10 (B and C) or 11 (D and E) and stained with monoclonal antibodies to rat β2 (B and D) and rhodamine–α-bungarotoxin (C and E) as in Fig. 2. Scale bar, 30 μm.

1C), these chimeras formed compact matrix-associated aggregates (Figs. 2F and 3B). All of these chimeras had β2 sequence in domains III to VI, which suggested the existence of a difference between β1 and β2 in this region that influences localization. To test this possibility, we generated chimeras 13 and 14, in which either domains III and IV or domains V and VI of β1 replaced the corresponding β2 domains in chimera 6 (Fig. 4A). Chimera 13 formed extracellular aggregates indistinguishable from those of chimeras 6, 8, 10, and 12 (Fig. 4B), whereas chimera 14 was distributed along fibrils in a pattern indistinguishable from that of native β1 (Fig. 4D). Thus, sequences in domain V or VI (or both) of β1 promote its association with fibrillar components of the extracellular matrix (*25*).

Our data provide evidence for two sites that influence the localization of laminin β chains: one in domain I, near the COOH-terminus of the protein, and another in domain V or VI, near the NH_2-terminus. A simple model is that a sequence in domain I of β2 promotes interaction with components of the postsynaptic apparatus, whereas a sequence in domain V or VI of β1 promotes interaction with components of the extracellular matrix (Fig. 1A). Although it is not clear how these sequences cause localization, some of the previously characterized interactions of laminin with cells and matrix components are mediated by binding sites that are within the localization domains we have defined here (*26*–*28*). For example, a non-integrin laminin-elastin receptor binds to a site within domain V of the β1 chain (*26*). This receptor is immunologically related to a galectin that is colocalized with laminin in C2 muscle cultures and may contribute to matrix assembly (*29*). This protein might bind less well to β2 than to β1. In addition, the α1 and γ1 chains of laminin bind to domains VI and V of β1, resulting in the polymerization of individual laminin-1 (α1β1γ1) trimers into a network (*28*). Corresponding domains of β2 may be unable to mediate polymerization with laminin-1.

With respect to the synaptic targeting sequence, the Leu-Arg-Glu (LRE) tripeptide at amino acids 1670 to 1672 is a major determinant of a site in β2 that is adhesive for motor neurons and inhibits neurite outgrowth (*8*). The 16–amino acid sequence required for synaptic localization (amino acids 1660 to 1675) includes this tripeptide sequence. In contrast, a laminin-1 fragment that includes the corresponding β1 region promotes neurite outgrowth (*27*). During regeneration after nerve injury, neurites grow along extrasynaptic basal lamina, which contains β1 but not β2, then stop growing and form synapses at original synaptic sites, which contain β2 but not β1 (*1*). Thus, sequences that mediate disparate functions of the β1 and β2 chains may also be responsible for their differential localization.

Finally, the targeting sequences identified here may be responsible for the nonoverlapping distributions of β1 and β2 that have been demonstrated in other cells and tissues (*5*, *6*, *30*, *31*). Although some cells may synthesize only β1 or β2, many synthesize both chains (*7*, *30*). In the latter cells, as in muscle, differential

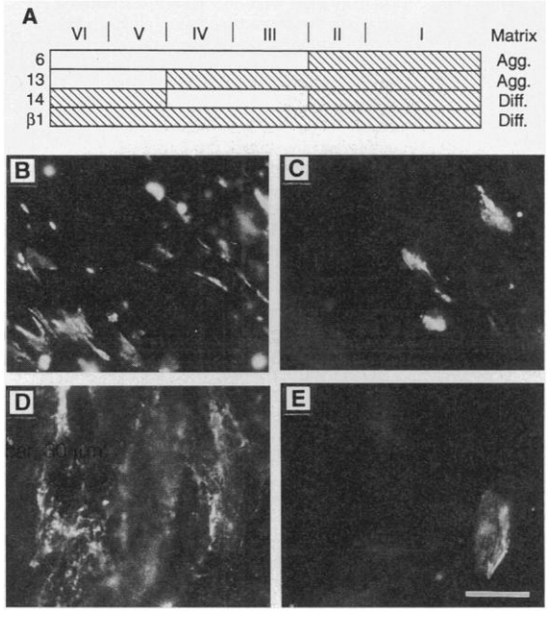

Fig. 4. Identification of a matrix localization domain in β1. (**A**) Comparison of chimeras 6, 13, and 14 with β1. All four proteins contain β1 sequences in domains I and II, but differ in having β1 sequences in neither, either, or both of the remaining two thirds of the molecule. Distribution of the proteins in C2 myotubes is indicated to the right: Agg., aggregated in intercellular areas; Diff., diffusely distributed along extracellular fibrils. (**B** to **E**) Cultures transfected with chimeras 13 (B and C) or 14 (D and E) and stained with monoclonal antibodies to rat β2 (B and D) and rhodamine–α-bungarotoxin (C and E) as in Fig. 2. Scale

association of the laminin β isoforms with cellular or extracellular components may determine their differential localization.

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14. Myotubes were incubated live with 5 nM monovalent rhodamine–α-bungarotoxin (Molecular Probes, Eugene, OR) in fusion medium for 1 hour. After washing with phosphate-buffered saline (PBS), cells were fixed in 1% paraformaldehyde for 25 min, washed with PBS, and incubated in PBS containing 10% goat serum for 1 hour. Cells were then incubated with primary antibody for 1 hour, washed once every 10 min for 40 min, incubated with the appropriate secondary antibody for 1 hour, and washed as before. For analysis of intracellular protein, fixed cells were permeabilized with 0.1% Triton X-100 for 10 min before addition of antibodies. Finally, cultures were mounted in glycerol with *p*-phenylenediamine and viewed on a microscope equipped with fluorescein- and rhodamine-selective filters. Antibodies have been described previously (*3, 5, 7, 15*). Immunoblot and immunohistochemical analysis showed that none of the monoclonal antibodies to laminin β2 used in this study (C1, C4, D5, D7, D19, D27, G1, and G6) cross-reacted with mouse antigen. Fluorescein-conjugated secondary antibodies were obtained from Boehringer-Mannheim (Indianapolis, IN).
15. Rotary shadowing showed that antibody 5A2 [D. R. Abrahamson *et al.*, *J. Cell Biol.* **109**, 3477 (1989)] binds to an epitope on either the β1 or the γ1 chain, and it stained mouse tissues in a pattern similar to that obtained with antibodies to β1 in rat and human tissues (*5*). We confirmed that 5A2 recognized the β1 chain by showing that it stained 3T3 fibroblasts transfected (*18*) with a laminin β1 expression vector (*31*) but not cells transfected with a laminin β2 expression vector (*7*).
16. The β1 chain is present at hot spots on cultured myotubes, as well as at synaptic sites on immature muscle fibers in vivo [A. Y. Chiu and J. R. Sanes, *Dev. Biol.* **103**, 4556 (1984)]. The loss of β1 is a late event in synaptic maturation in vivo and does not usually occur at hot spots in C2 cells.
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18. Cells were transfected with a calcium phosphate method [W. D. Phillips, M. M. Maimone, J. P. Merlie, *J. Cell Biol.* **115**, 1713 (1991)], then passaged the following day and selected in active G418 (800 μg/ml) (Gibco). Populations of stable transfectants were then fused to form myotubes or frozen for later use.
19. Chimeras 1 to 8 (Fig. 2) and 13 and 14 (Fig. 4) were constructed from full-length mouse laminin β1 (*31*) and rat laminin β2 cDNAs, with the expression vector previously described for β2 (*7*). Briefly, oligonucleotides with point mutations were used to generate fragments of β1 or β2 with a restriction site at a point of homology with an existing site in the other gene. The chimeras were then constructed by cutting both genes at that site. All sites maintained the same reading frame, except for Cla I, where a base was deleted at the β1-β2 junction to maintain the reading frame. The positions of junctions between β1 (in brackets) and β2 (in parentheses), as well as the locations of the restriction sites used in the construction, are as follows: chimera 1, (1–1599, Sfi I)/[1664–2526]/(Bsm I, 2457–5548); chimera 2, (1–2365, Stu I)/[2437–3715]/(Nsi I, 3649–5548); chimera 3, (1–1599, Sfi I)/[1664–3715]/(Nsi I, 3649–5548); chimera 4, [1–1662]/(Sfi I, 1599–5548); chimera 5, [1–3715]/(Nsi I, 3649–5548); chimera 6, (1–3649, Nsi I)/[3717–5643]; chimera 7, (1–3649, Nsi I)/[3717–5060, Cla I]/(4884–5548); chimera 8, (1–4884)/[Cla I, 5062–5643]; chimera 9, (1–4884)/[Cla I, 5062–5121]/(5058–5084)/[5149–5643]; chimera 10, (1–4884)/[Cla I, 5062–5175]/(5112–5144)/[5209–5643]; chimera 11, (1–4884)/[Cla I, 5062–5214]/(5151–5198)/[5263–5643]; chimera 12, (1–4884)/[Cla I, 5062–5307]/(5244–5264)/[5329–5643]; chimera 13, (1–1599, Sfi I)/[1664–5643]; chimera 14, [1–1599, Sfi I]/(1664–3715)/[Nsi I, 3649–5548]. Nucleotides are numbered as described previously (*5*) [M. Sasaki, S. Kato, K. Kohno, G. R. Martin, Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 935 (1987)].
20. For immunoblot analysis, transfected 3T3 fibroblasts were solubilized in 2% SDS and 70 mM β-mercaptoethanol, heated to 95°C for 3 min, separated on 6% denaturing polyacrylamide gels, and transferred to nitrocellulose. Nitrocellulose filters were incubated for 1 hour in tris-saline [10 mM tris (pH 8.0), 150 mM NaCl, and 0.2 mM EDTA] containing 0.1% Tween 20 and 5% nonfat dried milk, and then for 2 hours with primary antibody. After washing in tris-saline for 30 to 40 min, blots were incubated with alkaline phosphatase–conjugated secondary antibody (Boehringer-Mannheim) for 1 hour, washed again, and stained for alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate as substrate.
21. By staining (*14*) and immunoblot analysis (*20*) of transfectants, we were able to map the epitopes recognized by several previously described monoclonal antibodies to β2. The epitopes of C1, C4, D5, D7, and D27 are within domain I, D19 binds to domain III or IV, and G1 and G6 bind to domain V or VI. For C4, D5, and D7, these results are consistent with those obtained from bacterially expressed fragments (*3*). The distribution of chimeras in C2 cells was analyzed with those antibodies that recognized β2 sequences present in the chimeras.
22. For the construction of chimeras 9 to 12, a Cla I–Not I fragment that extended from a Cla I site to the Not I site in the 3′ polylinker was subcloned into pBluescript II-SK$^+$ (Stratagene). Oligonucleotides containing β2 sequences at their 5′ ends and β1 sequences at their 3′ ends were used to amplify this plasmid. Polymerase chain reaction products were isolated on a 1% agarose gel, purified, phosphorylated, and ligated, thereby generating Cla I–Not I fragments in which 7– to 16–amino acid segments of β2 replaced corresponding sequences in β1. Constructions were confirmed by DNA sequencing. The inserts were released with Cla I and Not I and cloned into chimera 8, from which the corresponding region of β1 had been removed with Cla I and Not I. For this purpose, chimera 8 was modified by destroying a second Cla I site in the vector. Oligonucleotides used were 5′-GGCATGTAACTGCCGAGCGCGCTGGGAGGCGTTGG and 5′-CTTCTGGAAGCCCTTAAGCGTAAAGCTGCCCAG (chimera 9), 5′-TGTAGATGCTGCCAGAGAGTTCTGGGCAGCTTTAC and 5′-GCTGAAGAGACAGCAGGCTCTGTAAAACAGAATGCAGACG (chimera 10), 5′-CTCAGCCTCCCTGGCACGGCTCT GTACAGAATATACTACTTTTTCG and 5′-AAACAGCTACGGGAACAAGTAGGTGAAAAGTATAAGAAGGTAGAAAG (chimera 11), and 5′-GGCCAGAACACCTTCAGTTTTTTGGGCAATTAAAC and 5′-GCACAAGCCAGAGCTGAGCTGCTACAAAATG (chimera 12).
23. Chimeras 1 to 8 and 10 to 14 were secreted, as judged by staining of nonpermeabilized transfected C2 cells (*14*). In contrast, little or no secretion of chimera 9 was detected by this assay.
24. We investigated whether the β1-β2 chimeras, like native β1 and β2, formed trimers with α and γ chains. First, we determined the apparent molecular mass of complexes containing chimera 11 (which localizes to hot spots) or chimera 10 (which does not) by immunoblot analysis. Both chimeras migrated as monomers (~200 kD) after reduction but as multimers (~800 kD) when reducing agent was omitted. The 800-kD band comigrated with authentic tumor-derived trimeric laminin-1. Second, we immunoprecipitated the chimeras with species-specific monoclonal antibodies to β2, then analyzed precipitates by immunoblotting. Precipitates from chimera-transfected (but not control) cells contained an ~800-kD complex stained by an antiserum to laminin-1 that recognizes α1, β1, and γ1, but not β2. Third, we immunoprecipitated laminins with a monoclonal antibody to the endogenous γ1 chain, and found that precipitates from chimera-transfected (but not control) cells contained an ~800-kD complex recognized by species-specific antibodies to β2. Together, these results demonstrate that the chimeras form heterotrimers with other laminin chains, including γ1. Moreover, a sequence in β1 that promotes chain assembly [A. Utani, M. Nomizu, R. Timpl, P. P. Roller, Y. Yamada, *J. Biol. Chem.* **269**, 19167 (1994)] is distinct from the synaptic localization sequence defined here. Reagents were not available to determine which α chains were present in the trimers, or whether different chimeras associated with different α chains.
25. Further support for the existence of an NH_2-terminal localization site was provided by reexamination of chimeras that were associated with hot spots. All chimeras with β2 sequences in the COOH-terminal localizing domain were confined to hot spots. However, those with β2 sequences in their NH_2-terminal one-third (chimeras 1 to 3, 7, and 11) and β2 itself were associated with most hot spots, whereas those with NH_2-terminal β1 sequences (chimeras 4 and 5) were absent from a substantial subset of hot spots.
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