A Novel Epitope of Entactin Is Present at the Mammalian Neuromuscular Junction

Arlene Y. Chiu and Jan Ko

Division of Neurosciences, Beckman Research Institute of the City of Hope, Duarte, California 91010

The extracellular matrix (ECM) at the neuromuscular junction (NMJ) is biochemically and functionally specialized, and bears molecules that can regulate both the formation and function of this peripheral synapse. We have previously purified one synaptic component of the muscle ECM—a unique laminin isoform named s-laminin—from a rat schwannoma cell line (Chiu et al., 1992). To develop new probes for the ECM, monoclonal antibodies were generated against other components produced by this cell line. One of these new antibodies, 9H6, binds selectively at the synaptic cleft of NMJs in adult rats, but not at extrasynaptic sites on the muscle surface. On Western blots, 9H6 recognizes a 150 kDa band that colocalizes, and copurifies with the laminin-binding, ECM glycoprotein entactin under both reducing and nonreducing conditions. N-terminal sequence analysis also indicates that the 9H6 antigen is related to entactin. However, polyclonal antibodies to entactin stain both synaptic and extrasynaptic sites. Thus, 9H6 appears to identify an entactin epitope with a very restricted distribution. Treatment with N-glycanase reduces the molecular mass of entactin and eliminates 9H6 binding, suggesting that the 9H6 epitope at synapses is dependent on glycosylation. Recent studies have shown that novel isoforms of laminin, collagen IV, agrin, and AChE are selectively sequestered at the NMJ. Our results indicate that the entactin present at the synaptic cleft also differs from entactin present outside the synapse. The synaptic form of entactin may contribute to the unique functions of the ECM at the neuromuscular synapse.

[Key words: extracellular matrix, entactin, s-laminin, basement membrane, neuromuscular junction, synapse specificity, carbohydrate epitope]

It has been known since the turn of the century that when motor neurons are injured, their axons return with great fidelity to reinnervate muscles at the original synaptic sites (Tello, 1907). It follows from these observations that physical constraints and environmental cues must guide the returning axons to the ap-propriate locations and promote the formation of new synapses. However, the molecules and mechanisms that regulate this process are just beginning to be understood. A major advance was the finding that extracellular molecules in the basement membrane at the neuromuscular junction (NMJ) can pinpoint sites where synaptic regeneration takes place (Sanes et al., 1978; Burden et al., 1979; Glicksman and Sanes, 1983; McMahan and Slater, 1984). These results prompted efforts to identify the relevant molecules, and study the processes by which such molecules can locally direct synapse formation.

A first step toward understanding the role of the extracellular matrix (ECM) in regulating synaptogenesis is to identify molecules unique to this region of the muscle basement membrane. Immunohistochemical studies (Sanes and Hall, 1979; Anderson and Fambrough, 1983; Sanes and Chiu, 1983), as well as studies with lectins (Sanes and Cheney, 1982; Ko, 1987; Ribera et al., 1987; Scott et al., 1988), demonstrate that synaptic ECM clearly differs biochemically from the ECM at extrasynaptic sites (reviewed in Sanes, 1989; Hall and Sanes, 1993). Moreover, a number of ECM molecules that have been implicated in the function and differentiation of the vertebrate NMJ are strategically located at the synaptic cleft. For example, the synaptic form of AChE is anchored to the basement membrane by a collagenous tail (Hall and Kelly, 1971; Hall, 1973; McMahan et al., 1978; Brandan et al., 1985; Krejci et al., 1991), where its activity is critical for normal and efficient synaptic function. A second example is agrin, a molecule released by motor neurons, that can induce focal accumulations of ACh receptors, and other postsynaptic specializations (Reist et al., 1992; reviewed in Nastuk and Fallon, 1993). Many forms of AChE and agrin are now known to exist; however, specialized, synaptic isoforms of these molecules are restricted to the synaptic cleft. A third ECM molecule that is preferentially sequestered within the synaptic cleft is the s-laminin chain, a homolog of the B1 laminin chain (Hunter et al., 1987, 1989a). In addition, antibodies specific for the A chain of laminin, and for certain subunits of collagen IV, also appear to bind selectively to the mammalian NMJ (Sanes et al., 1990). While the functions of these individual molecules are currently unknown, a larger picture of the organization of the ECM is beginning to emerge. Similar families of molecules make up the ECM at synaptic and extrasynaptic sites; however, the isoforms that are specifically expressed at the synaptic cleft differ from their homologs expressed over the rest of the muscle fiber surface. These synaptic isoforms, which may be unique polypeptides, or may have been altered by posttranslational modifications, are responsible for the distinct composition and function of the ECM at the NMJ. A new member of this group of

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Normal   ## Damaged

A. Without reduction
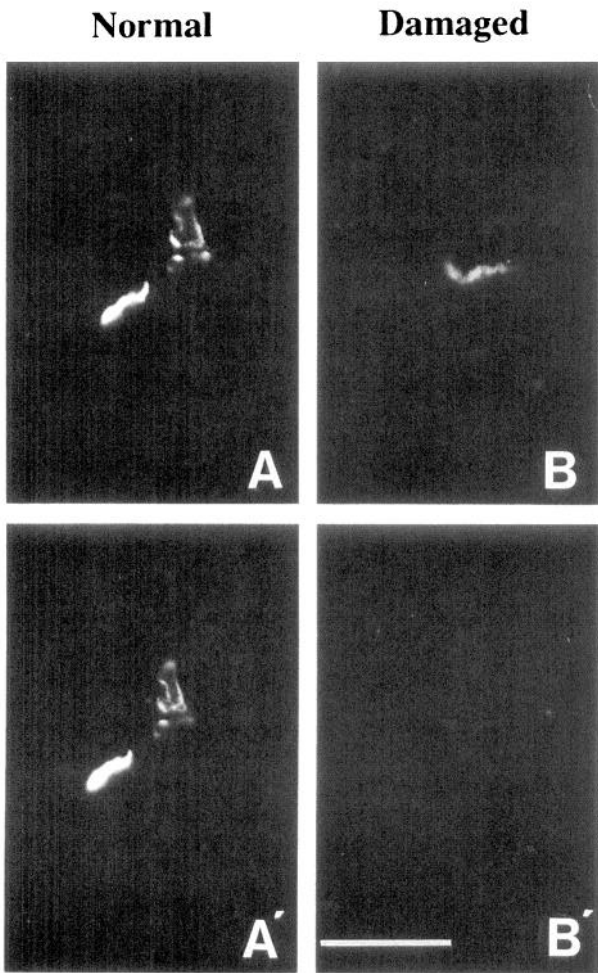

B. With reduction

Figure 1. Monoclonal antibody 9H6 specifically recognizes the ECM at NMJs. Flash-frozen sections of rat muscle were reacted with 9H6, followed by a fluoresceinated secondary antibody to visualize sites of binding. The sections were concomitantly labeled with rhodamine–α-bungarotoxin to identify end-plates. In normal muscles, antibody binding (*A*) coincided with toxin binding (*A´*). Following denervation and damage to the muscle, antibody binding remained with the ECM sheaths (*B*); however, no toxin-binding sites were left in the damaged muscles (*B´*). Scale bar, 50 μm.

synapse-specific ECM molecules is the laminin-binding glycoprotein entactin. In this article, we describe a novel epitope that distinguishes entactin at the rat NMJ.

Materials and Methods

Reagents. Serum-free medium was conditioned for 3–4 d by confluent cultures of the rat schwannoma, D6P2T, as previously described (Chiu et al., 1992); 1200 ml of conditioned medium was applied to a 70 ml bed of DEAE-Sephacel, and eluted with a 260 ml gradient of 10 mM to 1.2 M NaCl in 10 mM imidazole, pH 7.0. Fractions of 0.6 ml each were collected and analyzed for the presence of different ECM components by Western blots. Fractions with a high entactin content were pooled and concentrated fivefold in a Savant Speedvac concentrator before applying onto a 50 ml bed of 400 HR-Sephacryl, equilibrated in 10 mM imidazole, pH 7.0, with 10 mM NaCl. The first peak of protein was pooled and used for Western blot analyses or subjected to further purification by electrophoresis on low-melting-point agarose gels, as previously described (Chiu et al., 1992; Ugozzoli and Chiu, 1992).

Generation of monoclonal antibody 9H6. Schwannoma ECM components were purified and extracted from slices of agarose following electrophoresis; material that migrated with an apparent molecular mass of 185–140 kDa were pooled and injected into Balb/c mice to generate
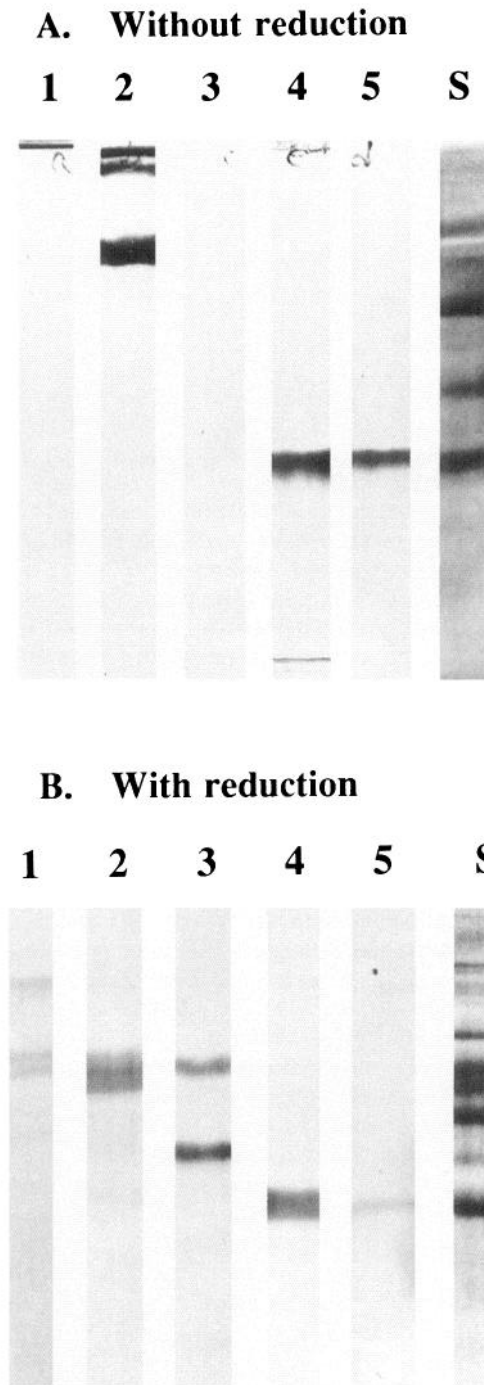
Figure 2. 9H6 recognizes a band that colocalizes with entactin on Western blots. Preparations of schwannoma ECM were separated by SDS-PAGE in the absence (*A*) and presence (*B*) of reduction, transferred onto nitrocellulose, and incubated with different antibodies to identify individual ECM components. The presence of tenascin (*lane 1*) and the B chains of laminin (*lane 2*) was observed with polyclonal antibodies. Monoclonal antibody C4 bound to both the B1 chain (205 kDa) and s-laminin (185 kDa) following reduction (*lane 3*). Polyclonal antibodies to entactin (*lane 4*) bound a band at 140–150 kDa that was also seen using monoclonal antibody 9H6 (*lane 5*). Silver staining revealed the protein content of each gel (*lane S*). The *arrowheads* indicate the relative migrations of molecular weight markers myosin (200 kDa) and phosphorylase b (92.5 kDa).
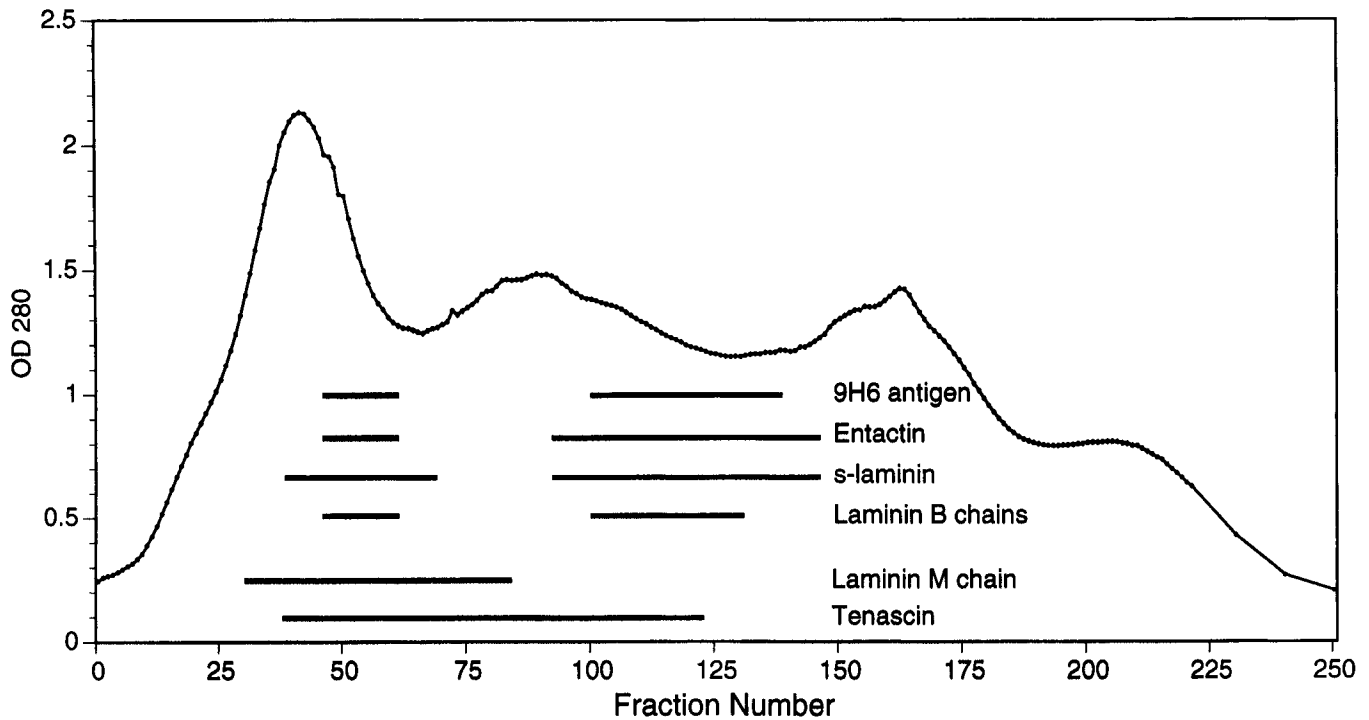
Figure 3. The 9H6 antigen copurifies with entactin. Over a liter of medium conditioned by the rat schwannoma D6P2T was separated by ion exchange chromatography and eluted with a gradient of NaCl. *Bars* indicate fractions bearing the highest concentrations of individual matrix components, as determined from Western blots.

monoclonal antibodies. Hybridoma supernatants were screened on sections of rat muscles, as previously described (Sanes and Chiu, 1983); end-plates were identified with rhodamine-labeled α-bungarotoxin. Hybridomas were subcloned if their antibodies bound selectively at end-plates.

Immunohistochemistry. Sections 6 μm thick of flash-frozen rat muscles and kidneys were cut on a Reichardt-Jung Frigo-Cut cryostat, picked up on alcohol-cleaned slides, and stored at −20°C till used. The following primary antibodies were used: 9H6 hybridoma supernatant, applied without dilution; ascites of monoclonal antibody C1, diluted 1:1000 with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween); rabbit anti-entactin (UBI, Lake Placid, NY) at 1:2500 dilution with PBS-Tween. After overnight incubation at room temperature in primary antibodies, sections were rinsed thrice in PBS-Tween before a 1 hr incubation in a mixture of the appropriate fluoresceinated secondary antibodies (1:100 dilution) and rhodamine-labeled α-bungarotoxin (1:3000 dilution; Molecular Probes, Eugene, OR). Goat anti-mouse IgM (Chemicon, Temecula, CA) was used to visualize 9H6 binding; goat anti-mouse IgG (Chemicon) for C1, and goat anti-rabbit IgG (Chemicon) for anti-entactin. The diluting buffer, PBS-Tween, was supplemented with 2% normal goat serum to reduce nonspecific binding. After rinsing off excess secondary antibodies, sections were examined under an Olympus IMT-2 fluorescence microscope equipped with separate filters for viewing fluorescein and rhodamine independently.

Polyacrylamide gel electrophoresis (PAGE) and Western blots. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed in 5.5% minigels, according to the method of Laemmli (1970). Transfer of gel-resolved proteins onto nitrocellulose was conducted as previously described (Chiu et al., 1991). Each blot was preincubated for 30 min in PBS containing 1% bovine serum albumin to block nonspecific binding prior to overnight incubation with primary antibodies at room temperature. Commercial polyclonal antibodies were used to identify tenascin (Chemicon), laminin (Chemicon), and entactin (UBI). Rabbit anti-human merosin was generously provided by Dr. E. Engvall (La Jolla Cancer Research Foundation, La Jolla, CA) and used to identify the rat M chain of laminin. All polyclonal antibodies were diluted with PBS-Tween. Hybridoma supernatant of 9H6, and of the monoclonal antibody C4, which recognizes s-laminin *in situ* (Sanes and Chiu, 1983), was used undiluted on Western blots, as previously described (Chiu et al., 1991). Following a 1 hr incubation in the appropriate secondary

antibody, conjugated to horseradish peroxidase and diluted 1:1000 with PBS-Tween, sites of binding were visualized with 4-chloronaphthol.

N-terminal sequence analysis. Approximately 250 μg of protein from schwannoma-conditioned medium, purified by ion exchange chromatography, gel filtration, and electrophoresis on agarose gels, was further separated by SDS-PAGE and transferred onto Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Adjacent lanes were reacted with 9H6 to identify the antigen; the immunoreactive band, visualized by Coomassie staining, was cut out and subjected to automated Edman degradation, using a sequenator built at the City of Hope (Hawke et al., 1985). phenylthiohydantoin-amino acids were detected by on-line HPLC systems.

Digestion with N-glycanase. For partial digestion with N-glycanase (Genzyme, Cambridge, MA), a sample containing 17 μg of schwannoma ECM, partially purified by ion exchange and gel filtration chromatography, was incubated in the presence of 0.65 U of recombinant N-glycanase overnight at 37°C prior to Western blot analysis (i.e., 23 μg/U enzyme). For more complete deglycosylation, 0.75 U of enzyme was used to digest 14 μg of sample (18.6 μg/U enzyme). In each case, the control sample was incubated without enzyme. To test the effect of deglycosylation on 9H6 immunoreactivity at NMJs, cryostat sections of rat muscles were incubated overnight at 37°C with N-glycanase (64 U/ml PBS) in a humidified chamber, then rinsed thrice in PBS before immunohistochemistry.

Results

Monoclonal antibody 9H6 recognizes an ECM component at the NMJ

To obtain new probes to ECM components, monoclonal antibodies were generated against matrix molecules partially purified from medium conditioned by the rat schwannoma D6P2T. Fresh-frozen sections of adult rat diaphragm were used in screening for antibodies that recognized synaptic components. One such monoclonal antibody, 9H6, selectively stained sites that were identified as end-plates by concurrent α-bungarotoxin binding (Fig. 1*A,A*). Since pre- and postsynaptic membranes, as well as the intervening ECM, are normally closely opposed
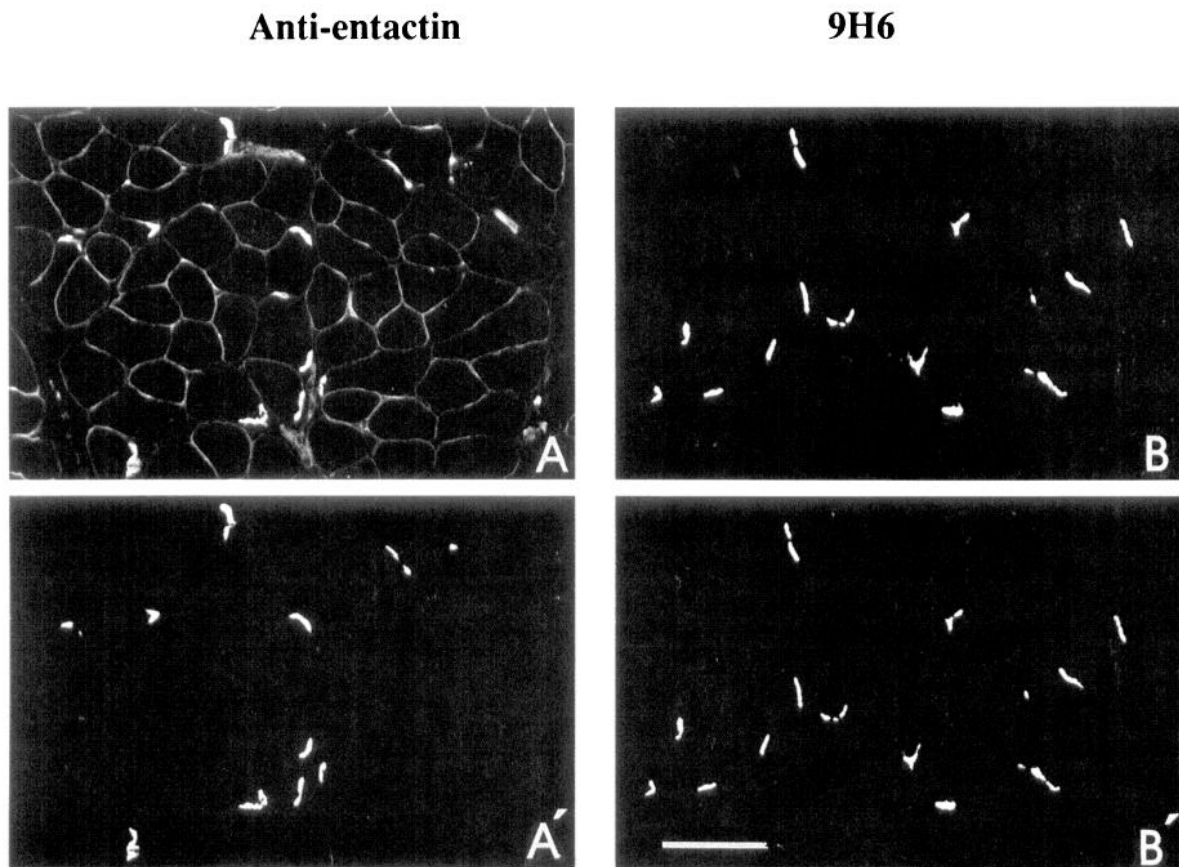
Figure 4. Comparison of 9H6 and anti-entactin immunoreactivity in rat muscle. The distribution of entactin (*A*) was viewed, using polyclonal antibodies, and compared with sites of 9H6 binding (*B*) in neighboring sections of rat muscle. Both types of antibodies stained synapses, as identified by rhodamine-labeled α-bungarotoxin in the same fields (*A'* and *B'*). However, anti-entactin also bound to extrasynaptic surfaces of muscle fibers. Scale bar, 200 μm.

at the NMJ, we needed a different preparation to determine where the immunoreactive epitope resided at this synapse. To resolve this issue, the sternomastoid muscle was denervated and damaged to eliminate both pre- and postsynaptic cells, leaving behind the "ghosts" of ECM sheaths (Sanes and Hall, 1979). In

sections of muscles damaged in this manner, the absence of α-bungarotoxin binding (Fig. 1*B'*) confirmed the loss of muscle membranes. In these preparations, immunoreactivity for 9H6 persisted at the original synaptic sites (Fig. 1*B*), indicating that the epitope is located in the basement membrane at the synaptic cleft.

Identification of the 9H6 antigen

Western blot analyses were employed to identify the antigen recognized by 9H6. Preparations of the original immunogen used to generate this antibody were separated by electrophoresis in the presence (Fig. 2*B*) or absence (Fig. 2*A*) of reducing agents, transferred onto nitrocellulose, and reacted with 9H6, as well as with a number of antibodies specific for known ECM molecules. Binding by 9H6 revealed a single, 150 kDa band that coincided with a band recognized by anti-entactin antibodies when electrophoresis took place without reduction (Fig. 2*A*, lanes 4, 5). This colocalization persisted in the presence of reducing agents (Fig. 2*B*, lanes 4, 5); however, 9H6 binding was diminished under these conditions. No binding by 9H6 was seen at bands identified by anti-tenascin (lane 1), anti-laminin (lane 2), or C4 (lane 3), a monoclonal antibody that reacts with both the s-laminin and the B1 laminin chains, on Western blots (Hunter et al., 1987, 1989a; Chiu et al., 1991, 1992). Other, unidentified proteins in the preparation could be seen with silver stain (lane S). Thus, the 9H6 antigen colocalized only with the entactin on Western blots.

Table 1. Tissue distribution of immunoreactivity for laminin and entactin

	Poly-clonal anti-laminin	Mono-clonal anti-body C1	Poly-clonal anti-entac-tin	Mono-clonal anti-body 9H6
Muscle				
Neuromuscular junctions	+++	+++	+++	+++
Myotendinous junctions	+++	+++	+++	+++
Extrajunctional sites	++	−	++	−
Intrafusal fibers	+++	+++	++	+++
Spindle capsule	+++	+++	++	−
Peripheral nerves				
Perineurium	+++	+++	++	−
Endoneurium	++	+	++	+
Kidney				
Glomeruli	+++	+++	++	−
Tubules	+++	−	++	−
Blood vessels	+++	+++	++	−

This 150 kDa, 9H6 antigen also copurified with entactin when schwannoma-conditioned medium was separated by ion exchange chromatography and gel filtration. More than 1 liter of conditioned medium was applied onto DEAE-Sephacel, and eluted with a gradient of 10 mM to 1.2 M NaCl, as previously described (Chiu et al., 1992). To determine the distribution of different ECM molecules, aliquots containing equivalent amounts of total protein from every fifth fraction were analyzed on Western blots. The 150 kDa, 9H6-immunoreactive band copurified with entactin, and was most prominent in fractions bearing the highest levels of entactin (Fig. 3). These fractions were also enriched for the smaller (185–220 kDa) laminin subunits: the B1, B2, and s-laminin chains. In contrast, immunoreactivity for the larger (300 kDa), M chain of laminin was only present in the first peak of entactin immunoreactivity, and the tenascin bands, most abundant in fractions 33–120, showed no meaningful overlap with the other ECM components examined. Entactin-enriched fractions were then separated by gel filtration chromatography. The 9H6 antigen and the entactin band eluted with an apparent molecular mass of greater than 1 million Da; again, both were found in the same fractions (data not shown).

When the purified 150 kDa, 9H6-immunoreactive band was subjected to Edman degradation, a single N-terminal sequence of 18 amino acids was obtained: NH_3-leu-asn-arg-gln-glu-leu-phe-pro-phe-gly-pro-gly-gln-X-asp-leu-glu-leu. This is an exact match of the N-terminal sequence of mouse entactin (Durkin et al., 1988), assuming a glycine is present at the X position.

If monoclonal antibody 9H6 recognized an epitope present on entactin, we reasoned that polyclonal antibodies to entactin should bind to histological sites identified by 9H6. In sections of rat muscle, anti-entactin stained both synaptic and extrasynaptic surfaces (Fig. 4*A*), although immunoreactivity was enhanced at end-plates. In contrast, 9H6 only bound to the ECM at the NMJ (Fig. 4*B*). In a survey of ECM from other tissues, entactin-like immunoreactivity was present at, but not restricted to, all sites where 9H6 bound (Table 1). Thus, polyclonal antibodies to entactin revealed a distribution that completely overlapped with, but was much more extensive than that seen with the monoclonal antibody 9H6.

The 9H6 epitope is destroyed by N-glycanase

In order to characterize the epitope recognized by 9H6, schwannoma ECM was digested with N-glycanase prior to Western blot analysis. With deglycosylation, entactin migrated to a lower position during electrophoresis (Fig. 5). At low enzyme concentrations, only some of the molecules were deglycosylated, and two bands were revealed with anti-entactin (Fig. 5*A*, lane 2). When sufficient enzyme was used, the 150 kDa band was lost, with a concomitant rise in the lower band, indicating that the lower band consisted of deglycosylated entactin (Fig. 5*B*, lane 2). Sister blots, reacted with 9H6, showed a reduction in the 150 kDa 9H6-immunoreactive band at low enzyme concentrations (Fig. 5*A*, lane 4). When N-glycanase concentration was increased, no immunoreactivity could be seen with 9H6 (Fig. 5*B*, lane 4). The deglycosylated form of entactin, identified by polyclonal antibodies, was not recognized by 9H6. The binding of 9H6 at end-plates was also vulnerable to deglycosylation (Fig. 6). Synaptic immunoreactivity was lost when sections of rat muscle were incubated in the presence of N-glycanase prior to immunohistochemistry. Thus, binding by 9H6, both on Western blots and in tissue, must require N-linked glycosylation of entactin.
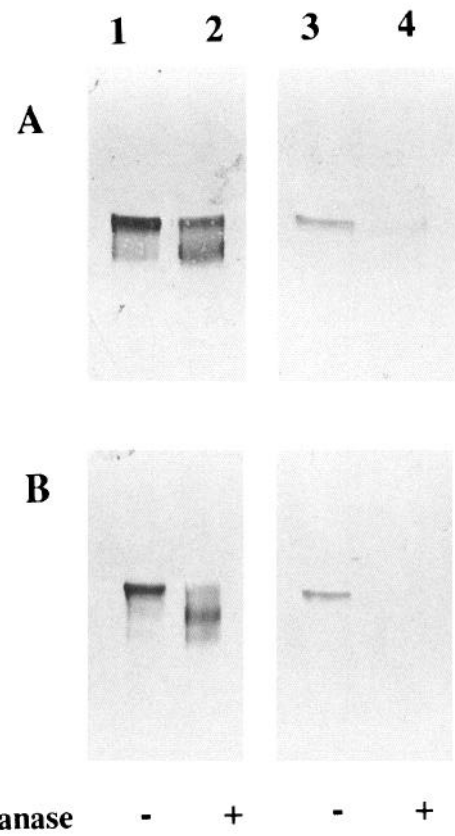

N-glycanase   -   +   -   +

Figure 5. N-glycanase digestion reduces the molecular mass of entactin, and eliminates 9H6 immunoreactivity on Western blots. An entactin-enriched preparation of schwannoma ECM was partially digested (*A*), at 23 µg protein/U N-glycanase, and more completely deglycosylated (*B*) at 18.6 µg protein/U enzyme. Control samples (*lanes 1 and 3*) were incubated in the absence of enzyme. Polyclonal anti-entactin antibodies (*lanes 1 and 2*) revealed that entactin migrated farther following removal of N-linked carbohydrates; the lower band increasing in intensity with increasing enzyme activity. The 9H6-immunoreactive band, which comigrates with entactin in the absence of enzyme (*lane 3*), is lost upon deglycosylation (*lane 4*).

Distribution of 9H6 immunoreactivity is much more restricted than that for s-laminin

Since the immunogen preparation that was used to generate 9H6 contained s-laminin, we sought to determine if the 9H6 epitope is present on s-laminin. However, the 150 kDa band recognized by 9H6 did not comigrate with s-laminin, which has a molecular mass of 185 kDa (Fig. 2*B*, lane 3). We also compared the distribution of s-laminin immunoreactivity with sites of binding by 9H6 in muscle ECM, as well as ECM from other tissues. Consecutive sections were stained with 9H6, and with a second monoclonal antibody, C1, which specifically recognizes s-laminin–rich sites (Sanes and Chiu, 1983; Chiu and Sanes, 1984). Both antibodies selectively stained end-plates (Fig. 7*A,A'*), myotendinous junctions (Fig. 7*B,B'*), and intrafusal muscle fibers (Fig. 7*C,C'*) in adult rat muscles. However, binding by 9H6 was conspicuously absent at a number of sites bearing high levels of s-laminin, including the muscle spindle sheath (Fig. 7*C'*), the perineurium surrounding peripheral nerves (Fig. 7*D'*), walls of blood vessels (Fig. 7*E'*, and arrows in Fig. 7*C'–E'*), as well as in kidney glomeruli (Fig. 7*F'*). Thus, the two patterns of immunoreactivity overlapped only on muscle ECM; the 9H6 epitope appears to have a more limited distribution than that seen for s-laminin (Table 1).
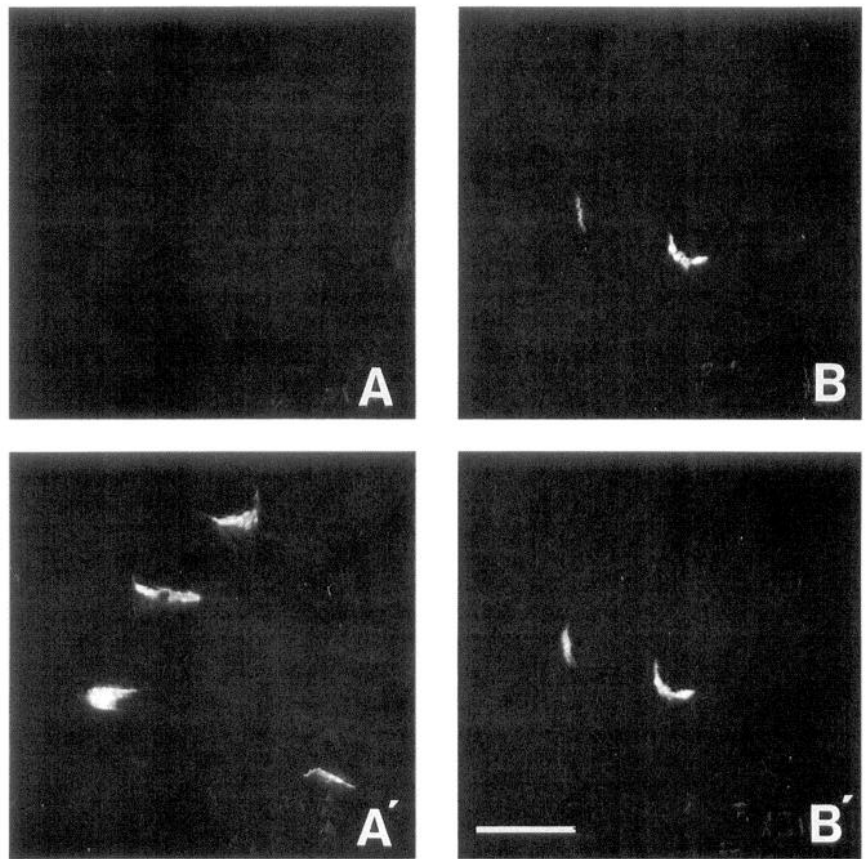
With N-glycanase   Without N-glycanase

Figure 6. N-glycanase eliminated immunoreactivity for 9H6 at NMJs. Cryostat sections of fresh frozen rat muscles were maintained at 37°C in the presence (*A*) or absence (*B*) of N-glycanase, prior to incubation with monoclonal antibody 9H6, and fluoresceinated secondary antibodies. End-plates were identified in the same field with rhodamine–α-bungarotoxin (*A'* and *B'*). Immunoreactivity for 9H6 was lost following deglycosylation (*A*). Scale bar, 100 μm.

Discussion

Since the ECM at the original site of the NMJ can direct synaptic regeneration (Sanes et al., 1978; Burden et al., 1979; Glicksman and Sanes, 1983; McMahan and Slater, 1984; Goldman et al., 1991; Brenner et al., 1992; Jo and Burden, 1992), considerable attention has been paid to identifying unique molecules localized to this region of the muscle ECM sheath. We have presented evidence that a novel epitope of entactin, recognized by a monoclonal antibody, 9H6, is highly concentrated or especially accessible at the rat NMJ. On Western blots, 9H6 binds to a single band of 150 kDa that comigrates with entactin, under both reducing and nonreducing conditions. This 9H6-immunoreactive antigen also copurifies with entactin. N-terminal sequence analysis of this band reveals a single sequence that is an exact match of the N-terminus of rodent entactin. Finally, N-glycanase digestion resulted in the loss of the 150 kDa band of entactin, as seen with polyclonal antibodies, and a concomitant elimination of 9H6 immunoreactivity on Western blots. The 9H6 epitope thus appears to be dependent on the glycosylation pattern of entactin. Since neuromuscular and myotendinous junctions are immunoreactive for 9H6, the entactin molecules present at these sites may differ from their extrajunctional counterparts in the carbohydrate moieties they bear. We cannot, however, rule out the alternative explanation that the 9H6 epitope is masked, and therefore unavailable for binding, at extrasynaptic sites. In either case, our results indicate that the entactin molecule differs between synaptic and extrasynaptic sites, and this difference resides in the pattern of glycosylation.

Synapse-specific carbohydrates have been described by several laboratories. Human, rabbit, rat, mouse, guinea pig, chick, frog, axolotl, snake, fish, and lamprey end-plates all bear high concentrations of a terminal N-acetylgalactosaminyl moiety that is recognized by two lectins, *Dolichos biflorus* agglutinin and *Vicia villosa-B₄* agglutinin (Sanes and Cheney, 1982; Ribera et al., 1987; Scott et al., 1988). These lectins bind to the asymmetric, collagen-tailed form of AChE, which is highly concentrated at synapses, but not to the more widely distributed globular forms of the enzyme (Scott et al., 1988). A lectin with a different specificity, peanut agglutinin, also binds selectively to the ECM at frog neuromuscular and myotendinous junctions (Ko, 1987, 1991), and appears to recognize a glycoconjugate of 30 kDa (Xiao et al., 1993). We have not as yet identified the carbohydrate moiety on entactin that is responsible for 9H6 immunoreactivity. Future experiments will enable us to determine if any of these lectins also bind to the entactin molecules at the NMJ. However, these lectin studies independently confirm the idea that differential glycosylation can confer a special identity to ECM molecules at synaptic sites.

The distinct nature of the basement membrane at the NMJ may arise from (1) differences in the carbohydrate composition of the resident molecules, (2) the presence of synaptic isoforms that have unique primary sequences, and (3) conformational changes in tertiary structure due to interactions with other ECM components that are specialized at the synaptic cleft. The synaptic forms of entactin and AChE are examples of molecules with carbohydrate specializations at the NMJ. S-laminin is an example of the second type of specialization. While immuno-
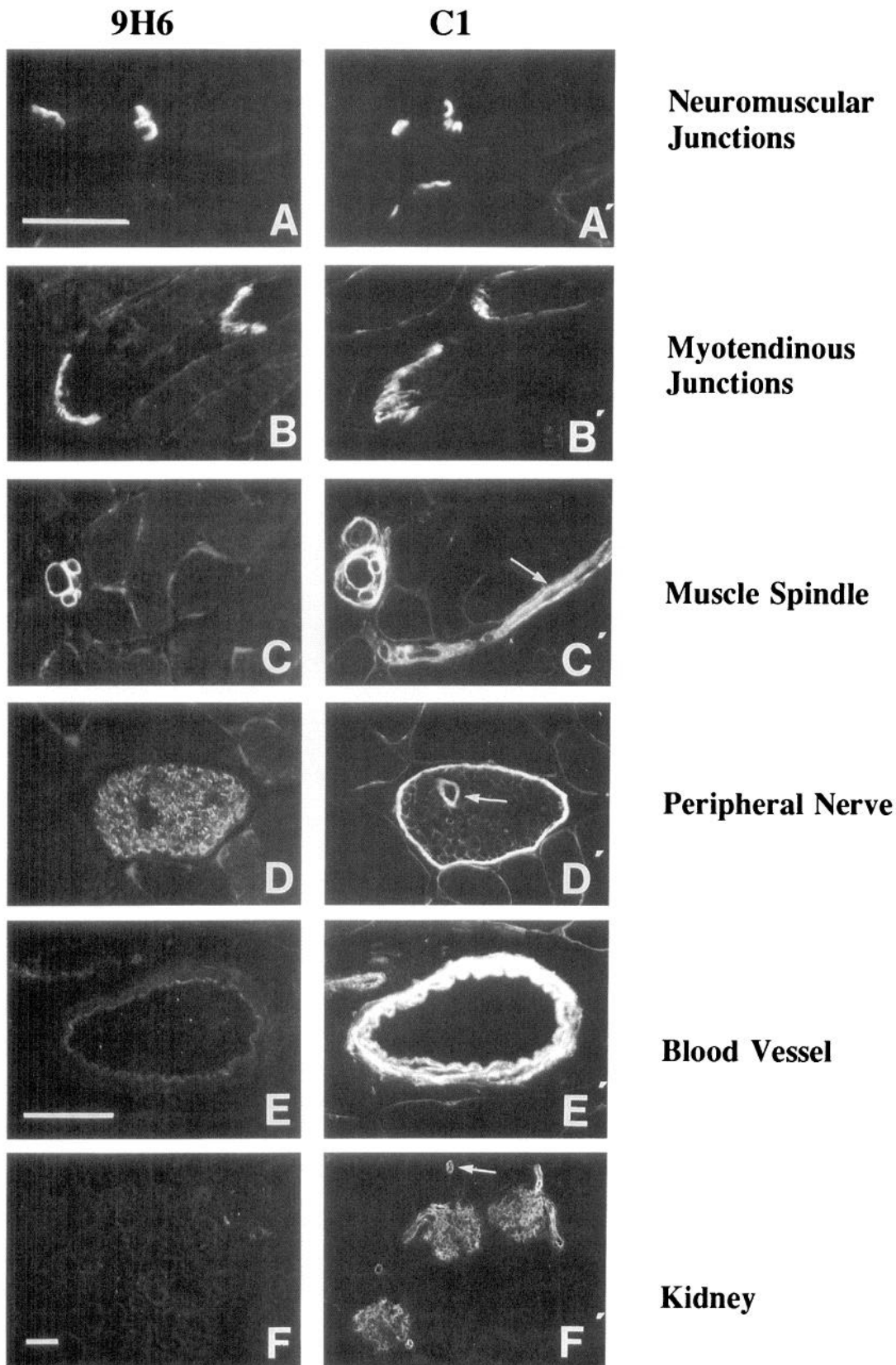
Figure 7. Immunoreactivity for 9H6 is more restricted than the distribution of s-laminin. Adjacent cryostat sections were reacted with 9H6 (*A–F*), or with another monoclonal antibody, C1, that recognizes s-laminin (*A′–F′*), and visualized with fluoresceinated secondary antibodies. Both monoclonal antibodies bound to end-plates (*A, A′*), myotendinous junctions (*B, B′*), and intrafusal muscle fibers (*C, C′*). Within peripheral nerves, 9H6 immunoreactivity decorated the endoneurium, on the abaxonal surfaces of Schwann cells (*D*). However, unlike C1, 9H6 did not bind to the muscle spindle capsule (*C*), the perineurium of peripheral nerves (*D*), blood vessels (*E* and the *arrows* in *C′, D′,* and *F′*), and kidney glomeruli (*F*). Sections in *B–E* share the same scale bar. Scale bars, 50 μm.

reactivity for s-laminin is concentrated at end-plates, B1 immunoreactivity is found everywhere on the muscle fiber surface, except at synapses (Sanes and Chiu, 1983; Engvall et al., 1990). Thus, the relatively rare s-laminin subunit replaces its more abundant homolog, the B1 chain, within the synaptic cleft.

Laminin molecules are believed to be organized as heterotrimers, each consisting of one long and two short chains. Currently, two long chains (the A and M chains) and three short subunits (the B1, B2, and s-laminin chains) have been described. Studies using chain-specific antibodies show that the s and B1 subunits can each be precipitated in the company of other laminin chains, but never together (Green et al., 1992; A. Y. Chiu, unpublished observations). These results indicate that the subunit composition of laminin at synapses must be different from laminin outside the synapse. Entactin is known to bind strongly with laminin. However, binding between the entactin molecule and the extrasynaptic, but not the synaptic, form of laminin may prevent the 9H6 antibody from recognizing its epitope. Thus, interaction with other ECM components could potentially alter tertiary structure, resulting in a conformational difference between synaptic and extrasynaptic molecules.

At present, a small number of matrix molecules that are selectively sequestered at the synaptic cleft have been identified, and each appears to serve a critical function at the NMJ. AChE maintains effective synaptic transmission. Agrin is likely to direct the organization of the postsynaptic apparatus on the muscle surface during development and regeneration (reviewed in McMahan et al., 1992; Nastuk and Fallon, 1993). S-laminin is preferentially adhesive to motor neurons (Hunter et al., 1989b, 1991), and may attract and keep motor axons at end-plates (Chiu and Sanes, 1984).

Entactin, also known as nidogen, is a dumbbell-shaped, sulfated glycoprotein that frequently copurifies with laminin (Timpl et al., 1983; Paulsson et al., 1987; see Fig. 3). A ubiquitous component of basement membranes, entactin displays many of the properties associated with other basement membrane proteins. For example, the preferential adhesion and migration of a number of cell types have been observed on entactin substrates *in vitro*, in part mediated by integrin recognition of an RGD sequence on entactin (Alstadt et al., 1987; Chakravarti et al., 1990; Senior et al., 1992; Yelian et al., 1993).

Although the function(s) of a synaptic form of entactin is currently not known, the very restricted distribution of the 9H6 antigen, even when compared with s-laminin (Fig. 7, Table 1), suggests a very specific function. Two possibilities present themselves. First, this molecule may prevent motor axons from sprouting beyond the end-plate, and initiate the formation and maintenance of a presynaptic terminal at this site. In support of this idea, there is evidence that the schwannoma RN22 produces a factor that inhibits neurite outgrowth on laminin substrates (Muir et al., 1989). Although the inhibitory molecule(s) has not been identified, we have found that schwannoma cell lines, such as RN22 and D6P2T, produce and release s-laminin (Chiu et al., 1991) as well as the 9H6 antigen. If ECM components, such as laminin and entactin, promote and guide neurons toward potential targets *in situ*, then the localized presence of specialized isoforms of these molecules may act to *terminate* axonal elongation and initiate synapse formation. A second possibility arises from the remarkable ability of entactin to bind and interlink laminin, collagen IV, fibronectin, heparan sulfate proteoglycans, and other components of the ECM (Dziadek et al., 1985; Aumailley et al., 1989, 1993; Mann et al., 1989; Chung

and Durkin, 1990; Wu and Chung, 1991; Wu et al., 1991; Battaglia et al., 1992; Senior et al., 1992). Since these ECM components interact independently with entactin, this promotes the formation of ternary complexes between laminin, collagen IV, and proteoglycans, and suggests a key role for entactin in the assembly and stabilization of basement membranes (reviewed in Chung and Durkin, 1990; Chung et al., 1993). Interestingly, synaptic isoforms of laminin (Hunter et al., 1987, 1989a), collagen IV (Sanes et al., 1990), and heparan sulfate proteoglycan (Anderson and Fambrough, 1983) are present at vertebrate end-plates. A specialized form of entactin at the NMJ may be the means by which the synaptic isoforms of many ECM molecules are selectively anchored to this critical site on the muscle surface.

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