Characterization of a 30-kDa Peripheral Nerve Glycoprotein That Binds Laminin and Heparin*

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The extracellular matrix glycoprotein laminin, a heterotrimer molecule made up of three chains of classes α, β, and γ, exerts important biological functions in various tissues (for recent reviews, see Refs. 1–5). In peripheral nerve, laminin plays crucial roles in both development and regeneration (6-12). Several different isoforms of laminin are expressed in different locations of peripheral nerve. In the endoneurium, laminin-2, comprising the α2, β1, and γ1 chains, is expressed surrounding nerve fibers, while laminin-1, comprising the α1, β1, and γ1 chains, is expressed surrounding blood vessels (13-17). The β2 and γ1 chains are expressed in the perineurium, but the α chain isoform of perineural laminin remains unknown (13-17). The fact that peripheral myelination is greatly disturbed in congenital muscular dystrophy patients and mice deficient in laminin α2 chain points to a specific role of endoneurial laminin-2 in peripheral myelogenesis (5, 18-29). Recent evidence also indicates a pathogenetic role of endoneurial laminin-2 in mediating the targeting of Mycobacterium leprae to Schwann cells in peripheral nerve (30).

Laminin exerts functions by interacting with various cell membrane and extracellular matrix proteins. Proteins that interact with laminin have been investigated extensively in various tissues or cell culture lines (for reviews, see Refs. 1–5, 31, and 32). However, studies aimed at the molecular dissection of these proteins in peripheral nerve are relatively few (15-17, 29, 33-37). Thus far, three protein assemblies, the dystroglycan, αβ1, and αβ4 integrin complexes, have been proposed as candidates for the Schwann cell receptors of endoneurial laminin-2 (16, 17, 29, 33-37). It is unclear, however, whether or how they mediate the myelogenesis induced by laminin-2.

Laminin is also known to interact with heparan sulfate proteoglycans (HSPGs) through multiple heparin-binding sites in the domain VI and the globular domain of the α chain (38-40). The interaction of laminin with HSPGs is important for both cell surface and basement membrane anchorage (39, 41). Moreover, the putative laminin-HSPG complex was shown to play an important role in the regeneration of peripheral nerve (6). Thus far, the cell surface and/or basement membrane HSPGs that interact with laminin have not been identified in peripheral nerve. Under these circumstances, it is of utmost importance to identify and characterize the proteins that interact with laminin in peripheral nerve.

Recently, we have demonstrated that, in addition to α-dystroglycan, bovine peripheral nerve contains a protein with a molecular mass of about 30 kDa that binds laminin in blot overlay assay (33). In this paper, we have characterized this 30-kDa bovine peripheral nerve laminin-binding protein, which we tentatively call LBP30.

EXPERIMENTAL PROCEDURES

Extraction of the Crude Bovine Peripheral Nerve Membranes at pH 12, by 0.5 M NaCl or 2% Triton X-100—Crude bovine peripheral nerve membranes, which contain the extracellular matrix proteins in addition...
to the membrane proteins, were prepared as described previously (16, 17, 33). Crude bovine peripheral nerve membranes were suspended at a protein concentration of 5 mg/ml in 50 mM Tris-HCl, pH 7.4, containing a mixture of protease inhibitors: benzamidine (0.75 mM), phenylmethylsulfonyl fluoride (0.1 mM), pepstatin A (0.7 mM), aprotinin (76.8 mM) and leupeptin (1.1 mM) (buffer A). For pH 12 extraction, the suspension was titrated to pH 12, extracted by gently stirring for 1 h or rigorously stirring for 2 h at room temperature, and centrifuged at 140,000 × g for 30 min at 25 °C. The supernatants were collected by decanting, cooled to 4 °C, and titrated to pH 7.4. The pellets were suspended in the original volume of buffer A. For pH 12 extraction, NaCl was added to the suspension to a final concentration of 0.5 M. The suspension was extracted for 1 h at 4 °C and centrifuged at 140,000 × g for 30 min at 4 °C. The supernatants were collected by decanting, and the pellets were suspended in the original volume of buffer A. For Triton X-100 extraction, Triton X-100 and NaCl was added to the suspension to a final concentration of 2% and 0.15 M, respectively. The suspension was extracted for 1 h at 4 °C and centrifuged at 140,000 × g for 30 min at 4 °C. The supernatants were collected by decanting, and the pellets were suspended in the original volume of buffer A.

Identification of the Peripheral Nerve Proteins That Bind to LBP30—The 0.8 M NaCl eluates (20 μg) of heparin affinity chromatography of the crude bovine peripheral nerve membranes were separated by 3–12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon). The PVDF transfers were blocked with 10 mM triethanolamine, pH 7.6, 140 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2 (LB) containing 5% nonfat dry milk (MLBB) and then incubated overnight two times at 4 °C with 10 ml of the pH 12 extracts of the crude bovine peripheral nerve membranes (5 mg/ml) in the presence of 1 mM CaCl2 and 1 mM MgCl2. After washing three times for 10 min at room temperature in MLBB, the proteins that bound to LBP30 on the PVDF transfers were detected with specific antibodies against various extracellular matrix ligands as described previously (33).

Identification of the Peripheral Nerve Proteins That Bind to Laminin—Mouse EHS sarcoma laminin-1 (10 nM) (34, Biomedical Technologies) was denatured in 65 mM Tris-HCl, pH 6.8, 3% SDS, 115 mM sucrose, 0.0004% bromophenol blue in the presence of either 1% β-mercaptoethanol (a reducing condition) or 10 mM N-ethylmaleimide (a nonreducing condition), separated by 3–12% SDS-PAGE, and transferred to PVDF membranes. The laminin PVDF transfers were blocked with MLBB and then incubated with 10 ml of the pH 12 extracts of the crude bovine peripheral nerve membranes (5 mg/ml) in the presence of 1 mM CaCl2 and 1 mM MgCl2 overnight two times at 4 °C. After washing three times for 10 min at room temperature in LBB, the proteins that bound to the laminin PVDF transfers were eluted with 2 ml of LBB containing 1 M NaCl and 10 mM EDTA.

Antibodies—Antisera were raised in Japanese white rabbits by injection, as immunogen, the 0.8 M NaCl eluates of heparin affinity chromatography of the crude bovine peripheral nerve membranes. Antibody against LBP30 was affinity-purified from the antisera using the LBP30 PVDF strip as described previously (42). The specificity of the affinity-purified antibody was confirmed by immunoblotting (data not shown). Monoclonal antibody 2D9 against the proximal portion of the G domain of human laminin α2 chain was described previously (43). Affinity-purified rabbit antibody against mouse EHS sarcoma laminin, affinity-purified rabbit antibody against human fibronectin, and monoclonal antibody against human collagen type IV were purchased from Sigma. Monoclonal antibody against β-dystroglycan was purchased from Novocastra. Monoclonal antibodies against human laminin α1, α2, β1, and γ1 chains were purchased from Life Technologies, Inc. Monoclonal antibody C4 against bovine laminin β2 chain was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD.

Miscellaneous Procedures—Immunohistochemical analysis, 3–12% SDS-PAGE, and immunoblotting were performed as described previously (16, 17). SDS-PAGE was performed in a reducing condition unless indicated otherwise. Extracellular matrix ligand overlay assay was performed as described previously (16, 17, 33). Briefly, laminin overlay was performed using either mouse EHS sarcoma laminin-1 (10 nM) or human placenta laminin-2 (10 nM) (43), and laminin-1 or -2 that bound to the proteins on the PVDF transfer was detected with affinity-purified rabbit antibody against mouse EHS sarcoma laminin. Fibronecin overlay was performed using bovine plasma fibronectin (10 nM, Sigma), and the fibronectin that bound to the proteins on the PVDF transfer was detected with affinity-purified rabbit antibody against human fibronectin. Collagen type IV overlay was performed using human collagen type IV (10 nM, Chemicon), and the collagen type IV that bound to the proteins on the PVDF transfer was detected with monoclonal antibody against human collagen type IV. Lectin overlay assay was performed using biotylated lectins (Seikagaku Corporation), followed by detection with ABC kit (Vector).

RESULTS

We have reported previously that, in addition to α-dystroglycan with a molecular mass of 120 kDa, bovine peripheral nerve contains two proteins, LBP30 and LBP320, with molecular masses of about 30 kDa and 320 kDa respectively, that bind laminin in blot overlay assay (Fig. 1a) (33). In this study, we aimed at characterizing LBP30 in bovine peripheral nerve. To gain insights into the subcellular localization of LBP30 in peripheral nerve, we extracted the crude bovine peripheral nerve membranes under various conditions. Previously, we have reported that LBP30 is not extracted when the crude peripheral nerve membranes are stirred gently at pH 12 for 1 h (Fig. 1a) (33). More recently, we have found that a substantial fraction of LBP30 is extracted when the crude peripheral nerve membranes are stirred rigorously at pH 12 for 2 h (Fig. 1b). On close inspection, LBP30 comprised multiple bands with two prominent uppermost bands of about 30 kDa (Fig. 1). LBP30 was also
extracted from the crude peripheral nerve membranes by 0.5 M NaCl but not 2% Triton X-100 (Fig. 2). This was in sharp contrast to β-dystroglycan, an integral membrane protein of Schwann cells (16, 17, 44), which was extracted from the crude peripheral nerve membranes by 2% Triton X-100 (Fig. 2) but not at pH 12 (data not shown) or by 0.5 M NaCl (Fig. 2). In addition, a substantial fraction of α-dystroglycan and LB320 were extracted from the crude peripheral nerve membranes under all these three conditions (Figs. 1 and 2).

Fig. 3 shows the results of heparin affinity chromatography of the 0.5 M NaCl extracts of the crude peripheral nerve membranes. Although laminin and agrin, which are both well established as heparin-binding proteins, did not quantitatively bind laminin-Sepharose in the presence of 0.5 M NaCl, LB320 was absorbed completely by heparin-Sepharose in this condition (Fig. 3, data not shown). LB320 was eluted from heparin-Sepharose by 0.5 M NaCl (Fig. 3).

To determine whether LB320 is glycospread, we stained PVDF transfers of the 0.8 M NaCl eluates of heparin affinity chromatography with various lectins. LB320 was stained positive with Concanavalin A, Lens culinaris lectin, wheat germ agglutinin, Phaseolus vulgaris E4, P. vulgaris L4, or Datura stramonium agglutinin (Table I). In contrast, LB320 was stained positively with Maackia amurensis agglutinin, peanut agglutinin, and Vicia villosa agglutinin isolecitin B4, which recognize sialic acid-linked α-2–3 to galactose, unsubstituted Ser/Thr-linked disaccharide Galβ1–3GalNAc unit, and nonreducing terminal β-GalNAc, respectively (Table I). These results indicate that LB320 contains both terminal sialylated and nonsialylated Ser/Thr-linked oinosaccharides. LB320 was also stained positive with Lotus tetragonolobus lectin and Ules europaeus agglutinin 1 (Table I), indicating that it contained fucose residues.

To identify the extracellular matrix ligands that bind to LB320, we overlaid the PVDF transfer of the 0.8 M NaCl eluates of heparin affinity chromatography with laminin-1, laminin-2, fibroenectin, or collagen type IV. Although LB320 bound laminin-2, as well as laminin-1, it did not bind fibroenectin or collagen type IV (Figs. 4a and 5). Previously, we have demonstrated that laminin heterotrimers are extracted from the crude peripheral nerve membranes at pH 12 (17, 33). To identify the endogenous peripheral nerve laminin isoforms that bind to LB320, we overlaid the PVDF transfers of the 0.8 M NaCl eluates of heparin affinity chromatography with the pH 12 extracts of the crude peripheral nerve membranes. All of the laminin chain isoforms known to be present in peripheral nerve, the α1, α2, β1, β2, and γ1 chains, were detected bound to LB320 (Fig. 4b).

We tested several reagents that might perturb the binding of LB320 to laminin. The binding of laminin-1 (Fig. 5) and laminin-2.
The higher molecular weight bands detected in lanes Anti-Lam and Anti-Lam 1 chain separated by 3–12% SDS-PAGE and transferred to PVDF membranes. The PVDF transfer was overlaid with laminin-1 in the absence (Control) or presence of 1 mg/ml heparin (Heparin), 1 mg/ml heparan sulfate (Heparan Sulfate), 1 mg/ml chondroitin sulfate (Chondroitin Sulfate), 10 mM EDTA (EDTA), or 1 M NaCl (1 M NaCl). The higher molecular weight bands are the endogenous peripheral nerve laminin chains that were present in the 0.8 M NaCl eluates of heparin affinity chromatography and reacted with antibody against laminin-1. Molecular size standards (Da × 10⁻³) are shown on the left.

To determine the localization of LBP30 in bovine peripheral nerve, we performed immunohistochemical analysis using affinity-purified rabbit antibody against LBP30. LBP30 was distributed broadly throughout the perineurium and endoneurium (Fig. 7). Thus, the distribution of LBP30 partially overlapped with that of laminin in bovine peripheral nerve.

**DISCUSSION**

Our results indicate that LBP30 is a laminin- and heparin-binding glycoprotein localized in the perineurium and endoneurium of bovine peripheral nerve. The molecular identity of
LBP30 remains unclear at present. LBP30 appears distinct from the two previously reported laminin-binding proteins that resemble LBP30 in size, the 32/67-kDa laminin-binding protein and carbohydrate-binding protein 35 (for reviews, see Refs. 1, 31, 32, and 45), for two reasons. 1) The 32/67-kDa laminin-binding protein and carbohydrate-binding protein 35, but not LBP30, can be extracted by detergents; and 2) the binding of LBP30 to laminin is not affected by the YIGSR peptide, galactose, or lactose. 2

Also remaining unclear is the molecular nature of the interaction of LBP30 with laminin. In contrast to α-dystroglycan, LBP30 bound to laminin-1 denatured by SDS in a nonreducing condition, indicating that the binding of LBP30 to laminin-1 is not as dependent on the high order structure of laminin as that of α-dystroglycan. However, the binding of LBP30 to laminin-1 was reduced drastically when laminin-1 was denatured by SDS in a reducing condition, indicating that this binding is somewhat dependent on the high order structure of laminin. In contrast to α-dystroglycan, in addition, the binding of LBP30 to laminin was not affected significantly by EDTA, indicating that the binding is not critically dependent on divalent cations. On the other hand, the binding of LBP30 to laminin was inhibited by heparin, heparan sulfate, or dextran sulfate, but was affected only mildly by chondroitin sulfate and not at all by dextran. These findings raise the possibility that the binding of LBP30 to laminin may be mediated by glycosylation.

The interaction of laminin with HSPGs through the heparin-binding sites located in the distal ends of both short and long arms of the α chain is important for both cell surface and basement membrane anchorage (38–41). The facts that 1) both LBP30 and laminin bind heparin, 2) heparin inhibits the binding of LBP30 with laminin, and 3) the distribution of LBP30 and laminin partially overlaps raise an intriguing possibility that LBP30 may modulate the biological activities of laminin by binding cooperatively and/or competitively to the same HSPG in peripheral nerve. In addition, LBP30 may indirectly modulate the interaction of laminin-2 with the Schwann cell dystroglycan complex, because HSPGs are known to differentially affect the interaction of various isoforms of laminin with α-dystroglycan (46).

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