

Isolation and Characterization of a Laminin-binding Protein from Rat and Chick Muscle

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Abstract. A major laminin-binding protein (LBP), distinct from previously described LBPs, has been isolated from chick and rat skeletal muscle (M_r 56,000 and 66,000, respectively). The purified LBPs from the two species were shown to be related antigenically and to have similar NH_2 -terminal amino acid sequences and total amino acid compositions. Protein blots using laminin and laminin fragments provided evidence that this LBP interacts with the major heparin-binding domain, E3, of laminin. Studies on the association of this LBP with muscle membrane fractions and reconstituted lipid vesicles indicate that this protein can interact with lipid bilayers and has properties of a peripheral, not an integral membrane protein. These

properties are consistent with its amino acid sequence, determined from cDNAs (Clegg et al., 1988). Examination by light and electron microscopy of the LBP antigen distribution in skeletal muscle indicated that the protein is localized primarily extracellularly, near the extracellular matrix and myotube plasmalemma. While a form of this LBP has been identified in heart muscle, it is present at low or undetectable levels in other tissues examined by immunocytochemistry indicating that it is probably a muscle-specific protein. As this protein is localized extracellularly and can bind to both membranes and laminin, it may mediate myotube interactions with the extracellular matrix.

INDIVIDUAL skeletal muscle fibers are surrounded by basement membranes which contain collagens IV and V, laminin, fibronectin, and proteoglycans (Sanes and Cheney, 1982; Kuhl et al., 1982). Although these components are common to all basement membranes, structural and compositional differences exist between the basement membranes of different tissues (Mohan and Spiro, 1986). In addition, the basement membranes within a tissue may exhibit anatomical specialization. For example, the muscle basal lamina is specialized in the region of the neuromuscular junction. The asymmetric forms of acetylcholinesterase and several other basement membrane molecules have a differential distribution between nonneuromuscular junction membrane and the neuromuscular junction (McMahan et al., 1978; Sanes and Hall, 1979; Sanes and Cheney, 1982; Sanes and Chiu, 1983). The muscle basal lamina accumulates during muscle development and is gradually remodeled to achieve an adult structure (Chiu and Sanes, 1984; Sanes et al., 1986). Experimental evidence suggests that myotube formation and interaction with both connective tissue elements and neuronal elements influences basal lamina formation (Anderson, 1986; Nitkin et al., 1983; Olwin and Hall, 1985; Sanderson et al., 1986; Sanes and Lawrence, 1983). Thus, the muscle basal lamina is a complex structure which is remodeled during development and has important effects upon the biology of the myotube and adjacent tissues.

Laminin, an abundant basement membrane component (Timpl et al., 1979), has been shown to potentiate cell attach-

ment and spreading, cell migration, and neurite outgrowth (reviewed in Liotta et al., 1986). Two distinct types of cell surface molecules have been postulated as mediators of cell interaction with laminin. They differ in apparent molecular weight and in affinity for laminin. Representatives of both types of laminin receptor have been demonstrated in skeletal muscle (Lesot et al., 1983; Horwitz et al., 1985). The lower affinity receptor was first identified by antibodies (CSAT and JG22) that blocked cell attachment to different extracellular matrix protein substrates (Greve and Gottlieb, 1982; Neff et al., 1982). These matrix receptors appear to be heterodimers, with apparent molecular masses between 110 and 200 kD, and are members of a super family of cell surface receptors, now named the integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Members of this family interact with a wide variety of extracellular matrix glycoproteins. They also contain cytoplasmic domains that bind proteins associated with the cytoskeleton (Horwitz et al., 1986). Individual integrins appear to be developmentally regulated in some tissues (Hall et al., 1987).

Members of the other major group of laminin-binding proteins (LPBs)¹, sometimes referred to as the high affinity receptors, bind to laminin with apparent affinities in the nanomolar range (Malinoff and Wicha, 1983; Terranova et al., 1983; Rao et al., 1983) and have apparent M_r of 68,000–72,000 when measured by SDS-PAGE in reducing condi-

1. Abbreviation used in this paper: LBP, laminin-binding protein.

tions (Lesot et al., 1983; Malinoff and Wicha, 1983; Terranova et al., 1983; Rao et al., 1983). These proteins have been isolated from murine melanoma cells (Rao et al., 1983), murine fibrosarcoma cells (Malinoff and Wicha, 1983), human breast carcinoma cells (Terranova et al., 1983; Barsky et al., 1984), as well as from skeletal muscle (Lesot et al., 1983) primarily by affinity chromatography on laminin. Monoclonal antibodies against the breast cell carcinoma laminin receptor inhibited the binding of radiolabeled laminin to carcinoma cells (von der Mark and Kuhl, 1985; Liotta et al., 1985) and polyclonal anti-laminin receptor antibodies appear to inhibit myoblast and melanoma cell attachment to laminin substrates (von der Mark and Kuhl, 1985; Wewer et al., 1987). In addition, a 120-kD membrane glycoprotein that is present in NG108-15 cells, chicken brain and mouse 3T3 fibroblasts interacts strongly with laminin and may represent a new type of cell surface/laminin-binding molecule (Smalheiser and Schwartz, 1987).

The goal of the present study was to purify large quantities of the major muscle LBPs to allow characterization of their distributions, laminin- and membrane-binding properties and cellular functions. A major LBP isolated by this procedure has properties that distinguish it from the two previously identified laminin receptors described above.

Materials and Methods

Materials

Adult White Leghorn chickens were obtained from Feather Hill Farm (Petaluma, CA). New Zealand White rabbits and rats were from Simonson Laboratories (Gilroy, CA). Affigel 10 Sepharose, and hydroxyapatite (HA) (DNA grade Bio-Gel HTP) were obtained from Bio-Rad Laboratories (Richmond, CA). Protein A Sepharose was from Pharmacia Fine Chemicals, Piscataway, NJ. DEAE-cellulose (DE-52) was purchased from the Whatman Company, (England). Thin layer chromatography plates and X-Omat film were from Kodak. En³Hance was from Dupont (Wilmington, DE). Trypsin (238 U/mg) and horseradish peroxidase-conjugated goat anti-rabbit antiserum were from Cooper Biomedical (Malvern, PA). Nitrocellulose filters were from Schleicher & Schuell (Keene, NH). Iodogen was from Pierce Chemical Company (Rockford, IL). Na¹²⁵I (16.7 mCi/ μ g) was from Amersham Corp. (Arlington Heights, IL) and [³⁵S]methionine was from ICN Radiochemicals (Irvine, CA). Egg lecithin was from Avanti Polar Lipids (Birmingham, AL). Gold-conjugated goat anti-rabbit antibody (AuroProbe EM GAR G20) was from Janssen Life Sciences Products (Piscataway, NJ). Lowicryl K4M was from Polysciences, Inc. (Warrington, PA). All other chemicals and biochemicals were from Sigma Chemical Company (St. Louis, MO).

LBP Purification

Extraction. Leg muscle tissue was homogenized in a Waring blender (3 \times 30 s) in 4 vol ice-cold homogenization buffer (10 mM Tris-HCl, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamide, and 1 mM EDTA, pH 7.4. All subsequent operations, except where noted, were performed at 4°C. The homogenate was filtered through one layer of gauze then centrifugated at 25,000 g for 30 min. The supernatant was discarded and the pellet resuspended in the same buffer by homogenization using a Polytron (2 \times 20 s). The sample was recentrifuged and the pellet dissolved (by Polytron) in the above buffer plus deoxycholate to a final concentration of 0.25%. After a 30-min incubation on ice the solution was centrifuged at 25,000 g for 45 min. The supernatant solution was saved and the pellet was extracted two additional times with the deoxycholate containing extraction buffer.

Ion Exchange Chromatography. The deoxycholate extract supernatants were pooled and the NaCl concentration was adjusted to 0.1 M by the addition of solid NaCl. This sample was applied to a DEAE-cellulose column (4.0 \times 8.0 cm) equilibrated with 10 mM Tris-HCl, 0.1 M NaCl and 0.1%

NP40, pH 7.4. The column was washed with 8–10 column volumes of the loading buffer and then with a buffer of identical composition except for a higher concentration of NaCl, 0.2 M and 0.25 M for columns loaded with rat and chick extracts respectively. The LBP was eluted from the DEAE-cellulose column with either 0.6 M NaCl (rat) or with 0.5 M NaCl (chick) at a flow rate of 30 ml/h. The last purification step differed for the rat and chick LBP: Concanavalin A chromatography for the rat LBP and hydroxylapatite chromatography for the chick LBP.

Concanavalin A Chromatography. Rat muscle LBP was further purified by chromatography on Concanavalin A Sepharose (1.0 \times 41.5 cm). The LBP recovered from the DE-52 column in 0.6 M NaCl was dialyzed into 10 mM Tris-HCl, 0.2 M NaCl, 0.1% NP40, pH 7.4 and applied to a Concanavalin A-Sepharose column equilibrated in the same buffer. The column was then washed extensively with the loading buffer. The LBP was eluted by rocking overnight in 10 mM Tris-HCl, 1 M NaCl, 0.1% NP40, 1 M α -methyl-D-mannopyranoside, pH 7.4, in a larger column (2.5 \times 15 cm). The eluate was dialyzed against 10 mM Tris-HCl, 0.15 M NaCl, 0.1% NP40, pH 7.4, concentrated by Amicon to 1 mg/ml, and was stored at -80°C .

Hydroxylapatite Chromatography. Chick muscle LBP was further purified by chromatography on hydroxylapatite at room temperature. The chick LBP recovered from the DEAE column in 0.5 M NaCl buffer was adjusted to a phosphate concentration of 0.2 M by the addition of 1.0 M NaPO₄, pH 7.4. It was then applied to an hydroxylapatite column (1.2 \times 6.0 cm) equilibrated in 0.2 M NaPO₄, pH 7.4, 0.1% NP40 at room temperature. The column was washed with at least 20 column volumes of loading buffer and was then eluted with a 200 ml 0.2–1.0 M NaPO₄, pH 7.4, linear gradient (20 ml/h). Fractions containing the chick LBP were dialyzed against 10 mM Tris-HCl, 0.15 M NaCl, 0.1% NP40, pH 7.4, concentrated to \sim 1 mg/ml, and stored at -80°C .

Preparation of Anti-LBP Antibodies

Anti-LBP antibodies were raised in female New Zealand White rabbits by standard protocols (primary immunization with 400–500 μ g of protein in Freund's complete adjuvant; secondary immunization at day 7 and every 30 d thereafter with 200–300 μ g antigen in Freund's incomplete adjuvant). Affinity purified anti-LBP antibodies were prepared by adsorption and elution with 3 M NaSCN using a 1 ml LBP-Affigel-10-Sepharose column containing \sim 1 mg LBP.

Immunohistochemistry and Immunoelectronmicroscopy

Frozen sections of rat skeletal muscle (5 μ m) were incubated with primary antibodies (15 μ g/ml) then peroxidase-conjugated second antibody, and diaminobenzidine by standard procedures (Graham and Karnovsky, 1966; Matthew et al., 1981). Controls, using affinity purified anti-P34 antibody, a major submaxillary gland protein of unknown function, and normal rabbit IgG were treated in the same manner. Rat heart and skeletal muscle were examined by post embedding staining of Lowicryl infiltrated sections using a modification of a previously described technique (Valentino et al., 1985). Sections were cut on a Reichart Ultracut E. Sections on grids were incubated with anti-LBP antibody (5 μ g/ml) or control antibody (anti-P34, 22 μ g/ml), rinsed, then incubated with gold-conjugated goat anti-rabbit IgG. The sections were viewed and photographed in a JEOL 100 B transmission electron microscope.

Immunoprecipitation of the LBP

Immunoprecipitation was performed using a modification of a previously described method (Kessler, 1981; John and Firestone, 1986). Extracts of metabolically labeled muscle plasma membranes, prepared as described below, were adjusted to 50 mM Tris-Cl, 150 mM NaCl, 0.5% NP40, pH 7.4 by the addition of concentrated reagents. Samples containing affinity purified anti-LBP antibodies (30 μ g/ml), preimmune serum, and samples without antibody were incubated at 4°C for 2 h. Samples were preincubated with Sepharose 4B and the Protein A-Sepharose was preincubated with unlabeled muscle extract to reduce nonspecific binding. After the samples were incubated with protein A-Sepharose for 1 h, the immunoprecipitates were collected by centrifugation and were washed four times with 50 mM Tris-Cl, 150 mM NaCl, 0.5% NP40, 0.05% SDS, 0.1% deoxycholate, pH 7.4. The pellets were dissolved in sample buffer and separated by SDS-PAGE. Gels were stained, destained, incubated in En³Hance then exposed to Kodak X-Omat film.

Protein Blots and Assays for ¹²⁵I-Laminin-binding and LBP Antigen

Laminin and laminin fragments were prepared as previously described (Timpl et al., 1979; Ott et al., 1982) and iodinated by the Iodogen method (Fraker and Speck, 1978). Proteins were separated by SDS-PAGE as previously described (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose sheets (0.2- μ m pore) in a Bio-Rad transfer apparatus using a current of 0.4 to 0.7 A for 2 h at 4°C (Towbin et al., 1979). Immunoblotting and protein blotting followed a modification of a previously described technique (Bixby and Reichardt, 1985). After transfer, nitrocellulose sheets were "blocked" by incubation in blocking solution (1% BSA and 0.4% hemoglobin in PBS; 100 mg/L CaCl₂, 200 mg/L KCl, 200 mg/L K₂SO₄, 100 mg/L MgCl₂ · 6 H₂O, 8 g/L NaCl, 2.16 g/L Na₂HPO₄ · 7 H₂O, pH 7.4) for 1 h at room temperature. After this blocking step the nitrocellulose was incubated with primary antibody (1/500 dilution of serum), or radioiodinated ligands (1.8 × 10⁷ cpm/ml laminin or laminin fragments) in blocking solution overnight at 4°C. The blots were then washed with 3 changes of PBS over 15 min. Protein blots were then dried and exposed to Kodak X-Omat film at -80°C. Protein blotting with radioiodinated laminin was used to determine the specific activity of the LBP at different steps of the purification. Units of LBP were calculated by comparing ¹²⁵I-laminin binding with various extracts or purified fractions to standard curves constructed by measurement of binding to known amounts of purified LBP. A unit of LBP is defined as 1 mg/ml of pure LBP. Immunoblots were incubated with a 1:1,000 dilution of peroxidase-labeled second antibody (goat anti-rabbit IgG) in blocking solution for 2 h at room temperature, then developed using H₂O₂ and 4-chloronaphthol as described by Hawkes et al. (1982).

Cell Culture and Radiolabeling

Primary chick myoblast cultures were prepared from breast muscle of day 11 chick embryos by the method of Antin et al. (1986). Cultures were maintained in MEM with Earle's salts containing 10% horse serum, 2.5% embryo extract, 50 U/ml penicillin and 50 μ g/ml streptomycin. Cells were plated on plates coated with 10 μ g/ml laminin and maintained in culture for 3 to 5 d. Cultures to be metabolically labeled were incubated overnight in methionine free culture medium containing 50-60 μ Ci/ml [³⁵S]methionine and supplemented with 0.2 mM methionine. At the end of the labeling period cells were washed three times with Ca⁺⁺, Mg⁺⁺ free PBS, then collected by centrifugation.

Preparation of Muscle Cell Plasma Membranes

Chick muscle cells in culture were metabolically labeled with [³⁵S]methionine as described above. The cell pellet was resuspended in 0.25 M sucrose, 10 mM triethylamine HCl, 1 mM PMSF, 1 mM N-ethylmaleimide, pH 7.4, and was homogenized in a Dounce homogenizer (Cates and Holland, 1978). The homogenate was centrifuged for 10 min at 1,700 g in a Sorvall SS-34 rotor. The supernatant was saved and the pellet was rehomogenized in 1/2 vol of the homogenization buffer and centrifuged. The supernatants were pooled and centrifuged for 1 h at 33,000 g. The pellet from this step was resuspended in the homogenization buffer and separated by a three step sucrose gradient (40, 32, and 17%, wt/vol) (Lesot et al., 1983). The gradient was centrifuged for 3 h at 130,000 g in a Beckman SW27 rotor. Fractions containing membranes were identified by OD₂₈₀ and peaks were pooled. The sucrose was diluted by the addition of triethylamine buffer without sucrose and membranes were collected by centrifugation at 150,000 g for 40 min at 4°C in a Beckman SW41 rotor. The membranes were aliquoted for different experimental treatments: 0.1 M NaOH, 4 M guanidine HCl, 20 mM lithium diiodosalicylate (LIS), 5 mM NaEDTA, 1 M NaCl, or 10 mM Na₂CO₃. Individual samples were adjusted to these concentrations, thoroughly mixed, and incubated at room temperature for 10 min. Samples were then centrifuged at 180,000 g in a Beckman airfuge to pellet the membranes. Supernatants and pellets were separately collected and immunoprecipitated with the anti-LBP antibody. After electrophoresis of the immunoprecipitates, fluorographs of the gels were scanned to quantify the relative amount of LBP in the supernatant and pellet from each sample.

Reconstitution of LBP with Artificial Liposomes

Protein-liposome interactions were measured as described previously (Doms et al., 1985). Liposomes were prepared by sonicating egg lecithin in 1 mM Tris-HCl, pH 7.5, for 15 to 20 min on ice (Racker et al., 1979).

Purified chick LBP, radioiodinated by the Iodogen method (Fraker and Speck, 1978), was added to 45 μ l of liposomes and incubated at room temperature for 1 h (5 μ l containing 2-20 μ g protein in 10 mM Tris-HCl, 0.1% NP40, pH 7.5). Different experimental agents were added at the end of this initial incubation and the protein liposome mixture was incubated for an additional hour at room temperature. The protein-liposome mixture was adjusted to 1.5 ml 45% sucrose and overlaid with 2.0 ml 30% sucrose, and 1.0 ml 10% sucrose. The samples were centrifuged at 200,000 g for 21 h at 4°C in a Beckman SW60 rotor. After centrifugation, fractions were collected and ¹²⁵I was quantitated in a Beckman Gamma 4000 counter.

Protein Partitioning in Triton X-114

The behavior of purified protein (LBP and two controls, glycophorin and BSA) in a 1% solution of Triton X-114 was determined by incubating ¹²⁵I-protein in 500 μ l of 1% Triton X-114, 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, at room temperature for 10 min. The two Triton X-114 phases were separated by centrifugation at 11,000 g for 3 min at room temperature and the relative concentration of ¹²⁵I-protein in each phase was determined in a Beckman Gamma 4000 counter.

Protein Analysis

Amino acid composition was performed on a Beckman 6300 amino acid analyzer as described for human insulin and IGF-I receptors (Fujita-Yamaguchi et al., 1986). NH₂-terminal sequence analysis was performed by Edman degradation on a multiphase sequencer (McLean et al., 1986). N-linked oligosaccharides were removed by overnight incubation at 30°C in the presence of 0.17% SDS, 50 mM NaEDTA, 1.25% NP40, 0.2 M NaPO₄, pH 8.6, and 10 U/ml N-glycosidase F. Protein concentrations were determined by the amido black method (Schaffner and Weissman, 1973).

Results

Purification of a Skeletal Muscle LBP

To identify major LBPs in skeletal muscle, rat and chick skeletal muscle extracts were fractionated by SDS-PAGE and blotted onto nitrocellulose. The blots were incubated with ¹²⁵I-laminin and examined for the presence of LBPs. Extraction and purification steps were optimized by following the LBP in this manner. The results revealed major LBPs of *M_r* 56,000 in chick muscle (Fig. 1) and *M_r* 66,000 in rat muscle (data not shown). These proteins did not bind detectable amounts of ¹²⁵I-fibronectin or ¹²⁵I-collagen IV using the same assay (data not shown). The most prominent LBP in each extract was purified in quantities sufficient to allow biochemical characterization (see Table I and Fig. 2). The most effective purification involved initial extraction of the muscle tissue in a buffer that did not solubilize the LBP and subsequent extraction of the insoluble residue with a deoxycholate containing buffer that did effectively extract the LBP. Deoxycholate extracted greater than 50% of the LBP present in the tissue; the remainder of the protein was present in an insoluble pellet, possibly associated with the extracellular matrix (Figs. 1 and 2). The deoxycholate extracts were chromatographed on DEAE-Cellulose. The LBPs from both chick and rat muscle were bound efficiently to DEAE-cellulose and were eluted with unusually high NaCl concentrations, resulting in more than 10-fold purification (Figs. 1 and 2; Table I). Rat LBP was eluted from DEAE-cellulose by 350 mM NaCl and chick LBP by 260 mM NaCl. Rat LBP was further purified by chromatography on Concanavalin A-Seph-rose and chick LBP was further purified by chromatography on hydroxylapatite. Table I shows the results of each purifi-

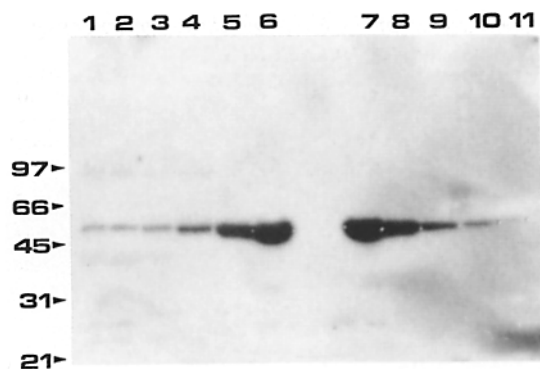


Figure 1. Autoradiograph of a ^{125}I -laminin protein blot. Purified avian LBP and samples from purification steps were separated by SDS-PAGE, electroblotted onto nitrocellulose, then blotted with ^{125}I -laminin as described in Materials and Methods. Muscle homogenate (lane 1), insoluble pellet (lane 2), deoxycholate extract (lane 3), DEAE-cellulose bound fraction (lane 4), hydroxylapatite bound fraction (lane 5), purified LBP (lane 6) are shown. Different amounts of purified LBP; 400, 200, 100, 50, and 25 ng are shown in lanes 7 through 11. The dried protein blot was exposed on Kodak X-Omat film. A photograph of the autoradiograph is shown. The position of molecular mass standards is shown on the left.

cation step for both the rat and chick muscle purifications. SDS-PAGE analysis of samples taken after each purification step for both rat and chick is shown in Fig. 2. Both rat and chick LBP were isolated to an estimated purity of greater than 90%, with purifications of LBP activity of 230- and 1,400-fold, respectively. From 100 g (wet weight) of muscle, the purification described above yielded 7.4 mg of the rat LBP and 4.7 mg of the chick LBP. As similar yields were obtained from each tissue, the difference in the purification factors probably reflects comparatively greater inactivation

of the rat LBP activity during purification. The results in Figs. 1 and 2 show that the purified rat and avian LBPs appeared to have the same relative molecular masses as the major LBPs detected in crude muscle extracts.

Characterization of Skeletal Muscle LBP

The purified LBP from rat muscle migrated on SDS-PAGE with an apparent M_r of 66,000 (Fig. 2, lane 5), similar in size to other previously described LBPs (Lesot et al., 1983; Malinoff and Wicha, 1983; Rao et al., 1983 and Terranova et al., 1983). The chick LBP has an apparent M_r of 56,000 (Fig. 2, lane 10). In spite of the difference in relative molecular masses, these two proteins were found to be homologous by several criteria. First, amino acid composition analysis demonstrated that both proteins had unusual, but very similar compositions (Table II). The abundance of Asn/Asp and Gln/Glu was particularly striking. Second, the NH_2 -terminal amino acid sequences of the purified chick and rat LBPs were determined and were found to be 60% identical for the first twenty amino acid residues (Table III). Computer analysis of the two sequences using the Dayhoff MDM-78 matrix resulted in a significant alignment score of 9.384 (Dayhoff, 1978). Thus, the chick and rat LBPs are very similar proteins. Finally, the proteins were also shown to be immunologically related. Polyclonal antisera raised against the rat muscle LBP cross reacted with the chick LBP (Fig. 3) and antisera raised against the chick LBP cross reacted with the rat muscle LBP (data not shown).

Tissue Distribution of the LBP

The affinity purified anti-LBP antibodies were also useful in determining the tissue distribution of the LBP. Tissue extracts from rat and chick liver, brain, kidney, retina, and heart were analyzed by SDS-PAGE and immunoblot with the anti-LBP antibodies (data not shown). This tissue survey

Table I. Purification Table

Rat muscle laminin-binding protein							
Purification step	Protein						Purification factor
	ml	mg/ml	mg	U/ml	U	U	
Homogenate	500	42	21,000	0.18	90	0.0043	1
Insoluble pellet	480	31	14,900	0.16	76.8	0.0052	1.2
DOC extract	800	2.7	2,200	0.07	56.0	0.025	5.5
DEAE effluent	45	1.1	49.5	0.81	36.5	0.74	172
Con A effluent	22.5	0.33	7.4	0.33	7.4	1.00	232
Chick muscle laminin-binding protein							
Purification step	Protein						Purification factor
	ml	mg/ml	mg	U/ml	U	U	
Homogenate	500	30.6	15,300	0.022	11	0.0008	1
Insoluble pellet	470	22.1	10,400	0.020	9.4	0.001	1.3
DOC extract	800	2.8	2,200	0.010	8.4	0.004	5.4
DEAE effluent	240	0.61	146	0.04	8.4	0.06	81
HAP effluent	18	0.26	4.7	0.26	4.7	1.00	1390

Purification Table for Rat and Chick LBP. Rat and chick muscle LBP were purified as described in Materials and Methods. At each major step of the two purifications, protein concentrations and laminin-binding activities were measured as described in Materials and Methods to obtain the specific activity in units/milligram protein. The purification factor was calculated by comparing the units of activity per milligram of protein in each fraction.

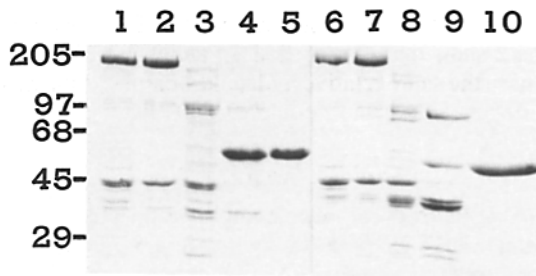


Figure 2. SDS-PAGE of proteins obtained at different steps of LBP purification. Proteins obtained at different purification steps of the rat (lanes 1-5) and chick (lanes 6-10) skeletal muscle LBP were separated by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue. Muscle homogenate (lanes 1 and 6), insoluble pellet (lanes 2 and 7), deoxycholate extract (lanes 3 and 8), DEAE-cellulose eluent (lanes 4 and 9) and either Concanavalin A eluent (lane 5) or hydroxylapatite eluent (lane 10) are shown. Molecular mass standards are indicated on the left.

demonstrated that only one other tissue, heart, had a detectable amount of anti-LBP antibody reactive protein. Similarly, when poly A⁺ RNA samples from the same chick tissues were analyzed for the presence of LBP mRNA, only skeletal muscle and heart were found to have high levels of LBP transcripts (Clegg et al., 1988). However, the heart protein had an apparent molecular mass of 56,000 in both rat and chick (Fig. 3). Thus, in rat the skeletal muscle and heart muscle forms of the LBP differed in apparent molecular mass (66,000 and 56,000, respectively), while in chick the two forms were of similar molecular mass (56,000). This difference is not due to differential N-glycosylation since treatment with N-glycosidase F reduced the apparent molecular mass of both forms by ~2,000 (Clegg et al., 1988).

To demonstrate the synthesis of LBP by muscle cells, em-

Table II. Amino Acid Composition of Rat and Chick LBP

Amino acid	Rat <i>mol %</i>	Chick <i>mol %</i>
Asp/Asn	19.7	20.0
Ser	3.2	3.2
Thr	3.9	3.8
Glu/Gln	18.3	15.0
Pro	5.1	4.2
Gly	5.0	6.2
Ala	7.0	5.2
Val	6.0	6.3
Met	1.2	1.3
Ile	4.8	7.4
Leu	8.8	8.3
Tyr	2.8	2.2
Phe	3.4	4.2
His	1.6	2.0
Lys	6.9	7.0
Arg	2.0	3.0

Amino acid composition of rat and chick LBP. The amino acid composition of the rat and chick skeletal muscle LBPs is shown in mole percent. The amino acid composition of the purified proteins was determined by hydrolysis and analysis on a Beckman 6300 amino acid analyzer as described in Materials and Methods.

Table III. Comparison of the NH₂-Terminal Sequences of the Rat and Chick Skeletal Muscle LBPs

	1	2	3	4	5	6	7	8	9	10
Rat	Glu	Asp	Gly	Leu	Asp	Phe	Pro	Glu	Tyr	Asp
Chick	Glu	Glu	Gly	Leu	Asn	Phe	Pro	Thr	Tyr	Asp
	11	12	13	14	15	16	17	18	19	20
Rat	Gly	Val	Asp	Arg	Val	Ile	Asn	Val	Ala	Asp
Chick	Gly	Lys	Asp	Arg	Val	Ile	Asp	Leu	Asn	Glu

The NH₂-terminal amino acid sequences of purified rat and chick skeletal muscle LBP were determined by Edman degradation on a multiphase sequencer.

bryonic chick skeletal muscle cells were grown in culture and metabolically labeled with [³⁵S]methionine. LBP was isolated from membrane fractions purified as above and immunoprecipitated with the anti-LBP antiserum. Results in Fig. 4 show the presence of a labeled band which comigrates with purified LBP and was specifically precipitated by the anti-LBP antiserum. Thus the chick skeletal muscle LBP is clearly synthesized by chick skeletal muscle cells.

The LBP is Localized Near the Plasma Membrane In the Extracellular Matrix and has Characteristics of a Peripheral Membrane Protein

Laminin is localized in the basement membrane which surrounds individual myotubes. To determine whether the LBP had a similar distribution, sections of rat muscle were immunostained with the anti-LBP antibody followed by a second antibody linked to peroxidase. The results, presented in Fig. 5, indicated that this LBP has a pericellular distribution similar to that of laminin (Sanes and Cheney, 1982). Exter-

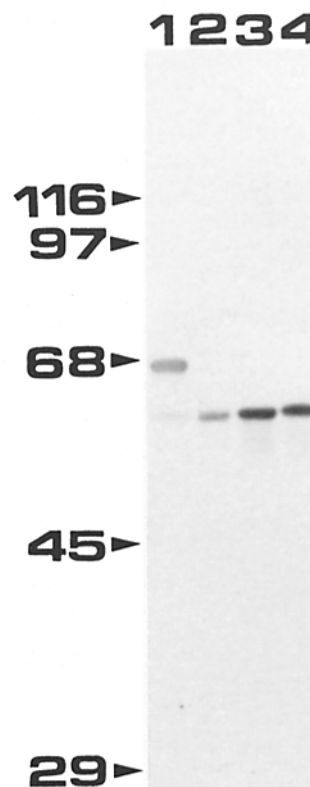


Figure 3. Immunoblot of rat and chick skeletal muscle LBP and rat and chick heart LBP. Rat and chick tissues were homogenized in electrophoresis sample buffer, boiled, centrifuged to remove particulate material then separated by electrophoresis on a 10% SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose and prepared for immunoblot as described in Materials and Methods. Affinity purified anti-rat LBP antibody was incubated with this blot and bound antibodies were detected as described in Materials and Methods. Rat skeletal muscle (lane 1), rat heart (lane 2), chick skeletal muscle (lane 3), and chick heart (lane 4) are shown.

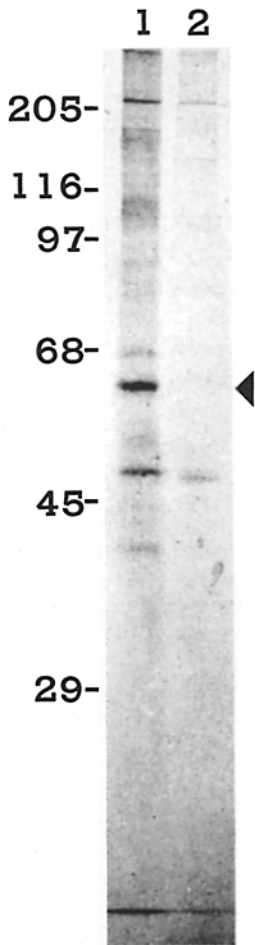


Figure 4. Skeletal muscle LBP is synthesized by muscle cells in culture. Chick myoblasts were maintained in culture and metabolically labeled with [³⁵S]methionine as described in Materials and Methods. Proteins were immunoprecipitated with anti-LBP antibody (lane 1) or preimmune serum (lane 2) and separated by SDS-PAGE (10% polyacrylamide, reducing conditions). A photograph of the fluorograph is shown. Molecular mass standards are indicated on the left.

nal radiolabeling with ¹²⁵I and immunoprecipitation confirmed an external surface localization for the LBP (data not shown). Immunoelectron microscopy showed that the LBP, detected using colloidal gold-conjugated IgG, was present extracellularly near the muscle plasma membrane and extracellular matrix (Fig. 6). It was not possible in this experiment to determine whether the rat LBP was primarily associated with the plasma membrane or the extracellular matrix.

The nature of the possible interaction of the LBP with the muscle plasma membrane was of particular interest since such a localization would place the LBP in a position to mediate cell interactions with laminin in the extracellular matrix or to modulate the interactions of laminin with other cell surface molecules. Two types of experiments were designed to analyze whether the LBP was associated with the muscle plasma membrane. The first involved determining whether purified muscle plasma membranes contained associated LBP and what the nature of the association was.

Purified muscle plasma membrane fractions were isolated by sucrose density gradient fractionation from cultured embryonic chick muscle cells metabolically labeled with [³⁵S]methionine (Cates and Holland, 1978). Neural cell adhesion molecule (NCAM), a known plasmalemma-associated protein, was detected in these fractions by immunoprecipitation with anti-NCAM antibodies (data not shown; Covault and Sanes, 1986). ³⁵S-LBP could be immunoprecipitated from these membranes with anti-LBP antibodies, showing that it was present in the purified chick myotube membrane fraction (see Fig. 4). To determine the nature of its association with these membranes, aliquots of purified muscle membrane were treated with reagents known to perturb protein-membrane interactions (Steck and Yu, 1973).

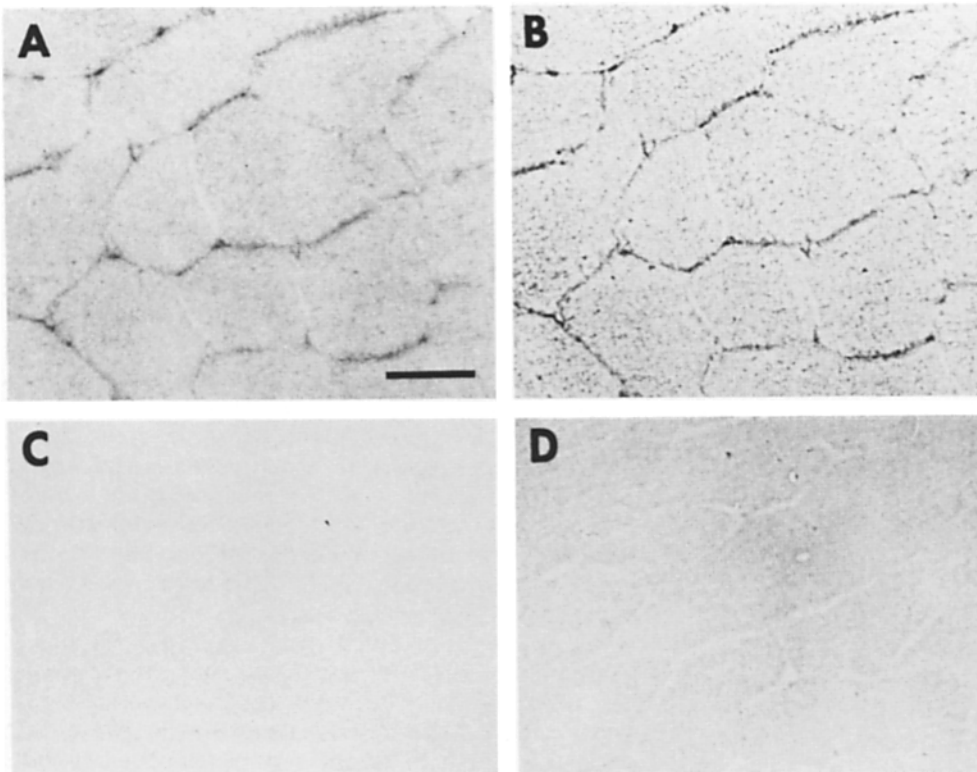


Figure 5. Photomicrographs of rat skeletal muscle sections stained with anti-LBP and control antibodies. Skeletal muscle was excised, quick frozen, sectioned on a freezing microtome and prepared for peroxidase immunocytochemistry as described in Materials and Methods. Muscle sections were photographed after incubation with either anti-LBP antibody (A, bright field; B, phase contrast) or, as a control, anti-P34 antibody (C, bright field; D, phase contrast). Note dark peroxidase staining product localizing the LBP between muscle fibers in the anti-LBP (A and B) but not in the control antibody (C and D) sections.

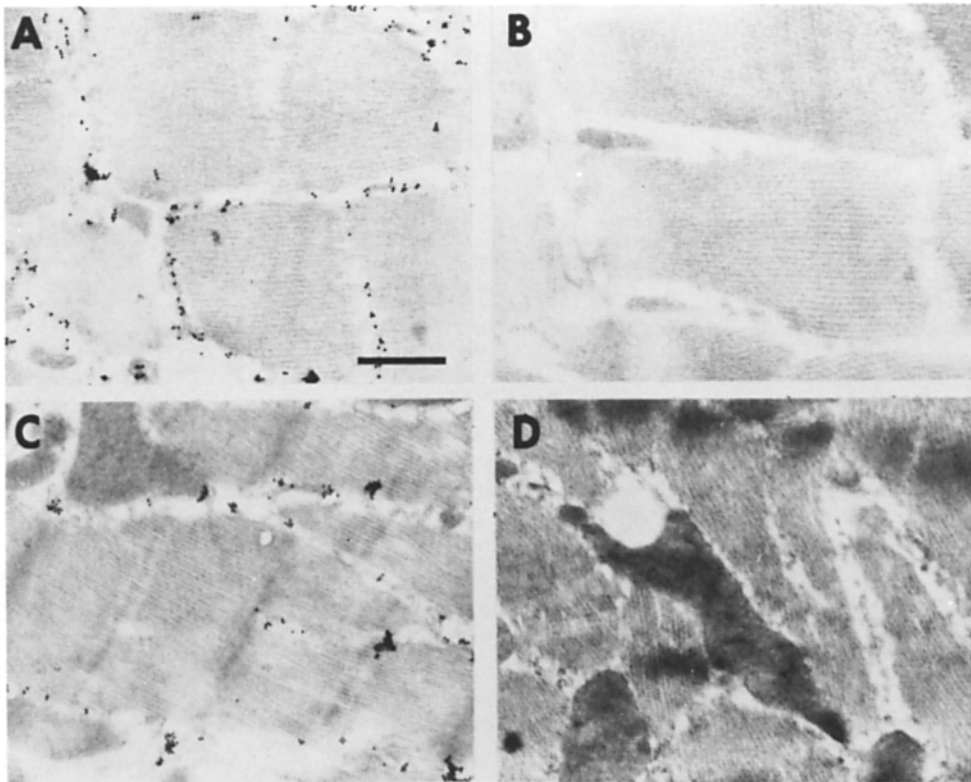


Figure 6. Electron micrographs of the rat skeletal muscle and heart reacted with anti-LBP antibody and visualized with colloidal gold-conjugated secondary antibody. Rat muscle was excised and processed for immunoelection microscopy as described (Materials and Methods). Skeletal muscle was incubated with either anti-LBP antibodies (A) or control antibody (anti-P34) (B); heart muscle was incubated with anti-LBP antibody (C) or control antibody (anti-P34) (D). Primary antibody was visualized by incubation with gold-conjugated goat anti-rabbit secondary antibody.

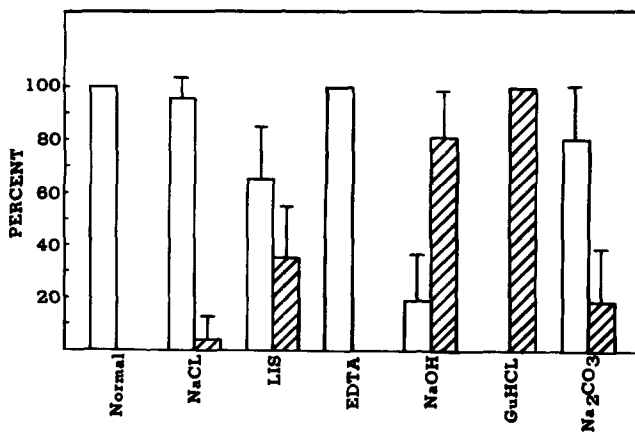


Figure 7. Determination of conditions which remove LBP from muscle membranes. Rat muscle membranes were isolated from metabolically labeled cells as described in Materials and Methods. Aliquots of these membranes were mixed with dissociative reagents including 1 M NaCl, 10 mM lithium iodosalicylate (LIS), 5 mM NaEDTA, 0.1 N NaOH, 4 M guanidine HCl (GuHCl) and 20 mM Na₂CO₃. Untreated and experimental membrane samples were centrifuged to separate membrane bound from solubilized LBP. The supernatants and pellets were separately collected and the LBP was quantified by immunoprecipitation with anti-LBP serum. The immunoprecipitates were separated by electrophoresis, gels were prepared for fluorography and exposed to Kodak X-Omat film. Fluorographs were scanned and the relative amount of LBP in the supernatant (cross-hatched bars) and pellet (open bars) were determined. The data shown is the average of three separate experiments, the error bars indicate SEM.

These included high pH (0.1 M NaOH), high ionic strength (1 M NaCl), a chaotropic agent (4 M guanidine HCl), a membrane perturbant (20 mM lithium diiodosalicylate, LIS), 5 mM NaEDTA and 10 mM Na₂CO₃. After treatment, the membranes were pelleted by centrifugation, then membrane associated and free ³⁵S-LBP were quantitated by immunoprecipitation with the anti-LBP antibody as described in Materials and Methods. The results of this analysis are shown in Fig. 7. LBP was not released from chick muscle membranes under the conditions of this assay unless perturbants were added. EDTA and high salt (1 M NaCl) did not release LBP suggesting that divalent cation-mediated and electrostatic interactions were not sufficient to account for the LBP-muscle membrane fraction association. Raising the pH in low ionic strength buffer (10 mM Na₂CO₃) released ~20% of the LBP while treatment with 0.1 N NaOH released ~80% of the membrane associated LBP. Treatment with 4 M guanidine HCl released 100% of the LBP to the supernatant. Thus, guanidine HCl was the most effective reagent for disrupting the LBP-muscle membrane fraction association.

In addition to determining what conditions resulted in LBP release from a purified muscle fraction, two other types of experiments were used to assess the hydrophobic character of the LBP. These included association with artificial liposomes (Racker et al., 1979) and Triton X-114 partitioning (Alcaraz et al., 1984). To examine the interaction of purified LBP with artificial lipid vesicles, the protein was radioiodinated by the Iodogen method and allowed to interact with artificial liposomes as described in Materials and Methods

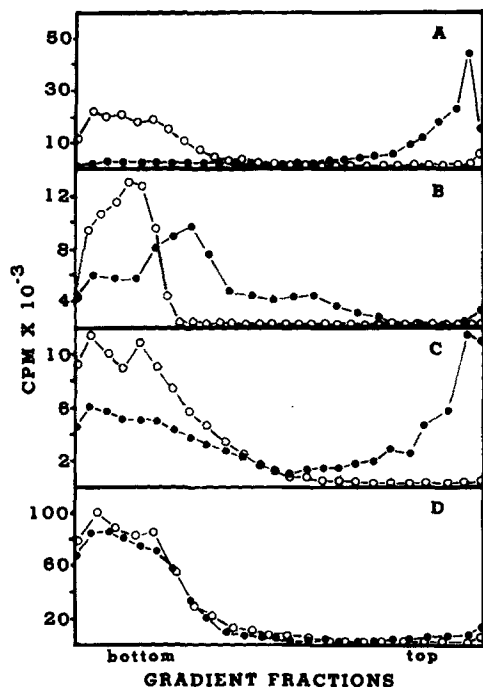


Figure 8. LBP interaction with artificial liposomes. Lecithin vesicles were prepared and incubated with radiiodinated proteins; purified LBP (A), glycophorin (C), or BSA (D). The protein liposome mixtures (closed circles) or protein alone (open circles) were then analyzed on discontinuous sucrose gradients (10, 30, and 45%) to separate liposome associated protein from nonassociated protein. B shows the effect of 0.1 N NaOH, pH 10.5–12, on the LBP/liposome association.

(Lesot et al., 1983). The liposome/protein mixture was then analyzed by sucrose gradient under conditions where liposomes with associated proteins would be well separated from unassociated protein. Glycophorin and BSA were used as positive and negative controls, respectively. The results show that approximately half of the purified glycophorin migrated with artificial liposomes (Fig. 8 C), as expected from the data of the other workers (MacDonald and MacDonald, 1975), while BSA did not associate with vesicles (Fig. 8 D). Approximately 65% of the total LBP added to the artificial liposomes migrated with the lipid vesicles (Fig. 8 A). Under no conditions did protein migrate near the top of the sucrose gradient unless liposomes were present (Fig. 8, A–D, open circles). Similar results have been obtained previously for a different LBP isolated from skeletal muscle (Lesot et al., 1983).

The interaction of chick LBP with artificial liposomes in the presence of various chemical perturbants was analyzed. As with natural muscle membranes, the LBP could be removed from artificial liposomes with 0.1 N NaOH (Fig. 8 B), whereas high salt (2 M KCl) was ineffective (data not shown). These findings indicate that there is a tight association between the LBP and the artificial liposomes and also indicate that the forces mediating this association are similar to those mediating the association of the LBP with natural membranes (cf. Fig. 7).

Partitioning of ^{125}I -LBP in detergent-poor and -rich phases of a Triton X-114 solution was analyzed as a measure of the hydrophobic nature of the native protein. Glycophorin

Table IV. Protein Partitioning in Triton X-114

	Detergent-poor phase (Hydrophilic)	Detergent-rich phase (Hydrophobic)
	%	%
LBP	90	10
Glycophorin	49	51
BSA	95	5

Radiiodinated purified proteins, LBP, BSA, or glycophorin, were mixed with Triton X-114 under conditions where partitioning into hydrophilic and hydrophobic Triton X-114 phases would occur (Alcaraz et al., 1984). The two phases were separated by centrifugation and the relative concentration of protein in each phase was determined by gamma counting. SDS-PAGE analysis of LBP recovered after the experiment showed that no degradation of the LBP occurred during the experiment.

and BSA were used as positive and negative controls respectively (Alcaraz et al., 1984). As expected, ~50% of the glycophorin was found in the lower hydrophobic phase while virtually all of the BSA was recovered in the hydrophilic, detergent poor phase (Table IV). Approximately 90% of the ^{125}I -LBP was recovered in the hydrophilic phase, indicating that the LBP surface is predominantly hydrophilic in nature (Alcaraz et al., 1984).

Laminin-binding Characteristics of LBP

Laminin is a multifunctional extracellular matrix protein and is known to interact with several other molecules, both on the cell surface and in the matrix. In some cases the precise laminin domain mediating these interactions has been identified (Rao et al., 1982; Terranova et al., 1983; Timpl et al., 1983; Edgar et al., 1984). Since the LBP interacted with laminin (see Fig. 1) but not collagen IV or fibronectin, in protein blots (data not shown), this method could also be used to identify laminin fragments that interacted with the LBP (Lesot et al., 1983). Laminin fragments prepared as previously described (Ott et al., 1982) were radiiodinated (Fraker and Speck, 1978) and used as ligands. The results presented in Fig. 9 show that laminin interacted strongly with purified rat (lane 2) and chick (lane 3) LBP and with rat LBP in a muscle extract sample (lane 1, Fig. 9). Laminin fragment E3 also interacted with chick LBP (lane 4) and rat LBP (lane 5) but laminin fragment E1, which contains a cell-binding domain for an M_r 68,000 laminin receptor (Rao et al., 1982; Timpl et al., 1983; Graf et al., 1987) did not (lanes 6 and 7). The failure of fragment E1 to interact with the LBP is unlikely to reflect denaturation, since this preparation was able to support the attachment of mouse mammary tumor epithelial cells and a number of other cells (Hall, D., C. Damsky, and L. F. Reichardt, unpublished observations).

Discussion

Homologous LBPs were isolated from rat and chick skeletal muscle by procedures that resulted in sufficient amounts of each protein for biochemical characterization. The purified protein bound laminin, but not fibronectin or collagen IV. Although the rat LBP purified by this procedure is similar in apparent molecular mass to LBPs from rat muscle (67,000; Lesot et al., 1983), murine fibrosarcoma cells (69,000; Malinoff and Wicha, 1983), murine melanoma (67,000; Rao et al., 1983) and human breast carcinoma cells

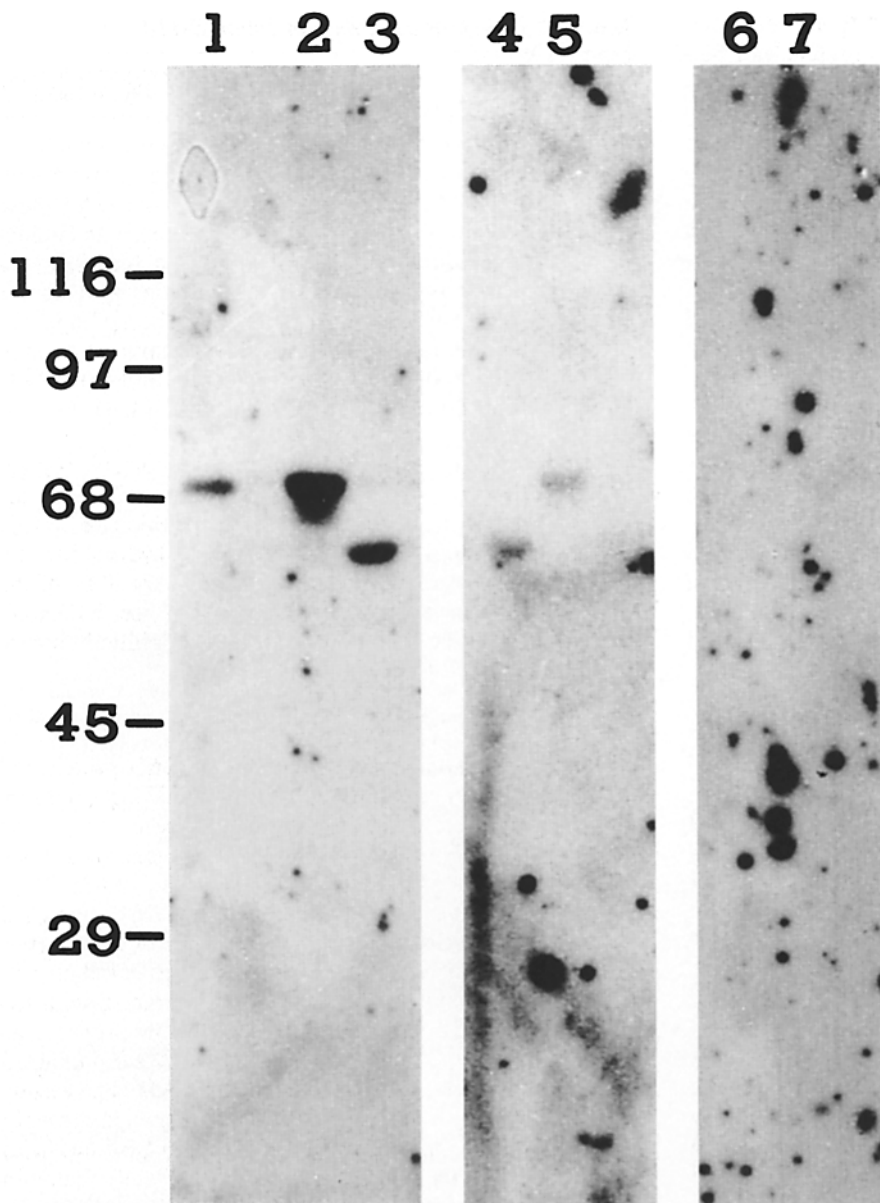


Figure 9 Interaction of skeletal muscle LBP with laminin. The figure shows an autoradiograph of a protein blot of LBP with ^{125}I -laminin and ^{125}I -laminin fragments. Rat skeletal muscle extract (lane 1), rat skeletal muscle LBP (lanes 2, 5, and 7) and chick skeletal muscle LBP (lanes 3, 4, and 6) were separated by SDS-PAGE, transferred to nitrocellulose. Sections of the nitrocellulose were incubated with ^{125}I -laminin (lanes 1-3), ^{125}I -E3 (lanes 4 and 5) or ^{125}I -E1 (lanes 6 and 7). Lanes 6 and 7 were exposed longer to ensure the absence of ligand binding.

(60,000-75,000; Terranova et al., 1983), it appears to be a different protein. First, the relative molecular mass of this LBP does not change under nonreducing conditions as does the murine fibrosarcoma cell laminin receptor (Malinoff and Wicha, 1983). Second, although previously described LBP from rat skeletal muscle has a high proportion of acidic amino acids and binds strongly to lipid vesicles, it has a very distinct amino acid composition (Lesot et al., 1983). Finally, comparison of a partial sequence of a laminin receptor isolated from human carcinoma cells (Wewer et al., 1986) with the complete sequence of the LBP that is the subject of this paper shows no similarity (Clegg et al., 1988). While this LBP is clearly different from these other proteins, it is not yet clear whether they share significant homologies.

Analysis of the amino acid composition of the chick and rat LBPs (Table II) and nucleic acid sequence analysis of two cDNAs encoding the chick LBP (Clegg et al., 1988) indicated that both rat and chick LBP have high negative charge

densities. Both the chick and rat LBP have high affinity for DEAE-cellulose and hydroxylapatite. In addition, the avian LBP has a long COOH-terminal poly-asp sequence that would be extremely anionic (Clegg et al., 1988). It will be interesting to determine whether this sequence is important in promoting binding of LBP to laminin at the E3 domain which is known to contain a site capable of binding the polyanion heparin (Ott et al., 1982).

The LBPs that were independently isolated from chick and rat skeletal muscle were shown to be homologous by tryptic peptide mapping, amino acid composition, and by NH_2 -terminal sequence analyses. The proteins, however, are not identical and differ in apparent molecular mass. Heart was the only other tissue that had a protein that was recognized by the anti-LBP antiserum. The heart LBPs from both rat and chick, had a relative molecular mass of $\sim 60,000$. Immunochemistry and immunoelectron microscopy demonstrated a similar distribution for the heart and skeletal muscle LBP's

(Figs. 5 and 6). The failure to detect the LBP in several non-muscle tissues examined suggests that this protein may be a muscle-specific protein. The RNA encoding this protein is also primarily found in skeletal and cardiac muscle (Clegg et al., 1988).

Several lines of evidence suggest that the LBPs in this paper are localized in the extracellular matrix in close association with the plasma membrane. The LBP is present in purified muscle membrane fractions, and is efficiently extracted by either detergent or by 4 M GuHCl, an effective extraction reagent for extracellular matrix proteins. Light and electron microscopy of muscle sections immunostained with the anti-LBP antibody suggested an extracellular distribution, though resolution was not sufficient to distinguish between a distribution in the muscle plasma membrane or the extracellular matrix (Figs. 5 and 6).

The LBP has properties consistent with an association with the muscle plasma membrane, characteristic of a peripheral, but not an integral, membrane protein. Purified muscle membranes containing the LBP, were treated with a variety of agents known to disrupt different membrane/protein interactions. The LBP was removed by high pH and chaotrophs, reagents that would not remove proteins intercalated in the lipid bilayer. Experiments with artificial liposomes showed that purified LBP interacted with lipid vesicles and was removed by high pH but not high salt. These experiments suggest that similar interactions are responsible for the association of LBP with muscle membranes and with liposomes. Assays of surface hydrophobicity by partitioning of LBP in Triton X-114 demonstrated that the LBP did not partition into the hydrophobic phase. Thus the LBP has an unusual combination of properties: it interacts strongly with liposomes and natural membranes but partitions in the aqueous phase of Triton X-114. SDS-PAGE analysis of the LBP recovered from the Triton X-114 experiment showed that the protein had not been degraded (data not shown). Furthermore, there is precedence for membrane proteins to partition in the aqueous phase of Triton X-114 (Maher and Singer, 1985). The hydrophilic nature of the LBP, suggested by Triton X-114 partitioning, and lack of a hydrophobic amino acid sequence long enough to serve as a transmembrane domain (Clegg et al., 1988) are consistent with a peripheral, but not an integral membrane localization. These experiments suggest that the LBP binds the muscle plasmalemma and the extracellular matrix protein, laminin.

Radioiodinated laminin fragments were used to determine which laminin domain interacted with the LBP. Laminin fragment E3, containing the heparin binding domain (Ott et al., 1982), but not laminin fragment E1 containing a cell-binding domain (Timpl et al., 1983; Rao et al., 1982) was found to interact with the LBP in protein blots. Substrate bound E1 fragment was able to support tumor cell attachment indicating that the fragment was biologically active (data not shown). The interaction of the LBP with laminin is probably not via the unusual gal α 1-3 gal carbohydrate found on murine laminin, since the LBP failed to bind to an affinity matrix containing this carbohydrate (data not shown). The LBP has a very high negative charge density and may therefore interact with what has been called the heparin-binding domain of the laminin molecule. This site of interaction also distinguishes the protein from a previously described laminin receptor which appears to bind a do-

main in a short arm (B1 chain) of the laminin molecule (Graf et al., 1987).

The rat and chick LBPs described in this paper are muscle proteins that bind laminin and are distributed extracellularly in the tissue near the plasma membrane and extracellular matrix. Thus they are proteins well placed to interact with laminin *in vivo*. Presently, no function is known for these LBPs though they may mediate cell interactions with laminin in the extracellular matrix, performing the function of a laminin receptor as proposed for other proteins with similar characteristics. Alternatively, the LBP could be a protein that binds to laminin and modulates the interaction of laminin with other matrix or cell surface molecules in the microenvironment near the cell surface or in the muscle basal lamina.

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