EXPERIMENTAL CELL RESEARCH **240,** 86–94 (1998)
ARTICLE NO. EX984002

Interaction of Integrin $\alpha7\beta1$ in C2C12 Myotubes and in Solution with Laminin

Anna Zolkiewska,[1] Walter C. Thompson, and Joel Moss[2]

Pulmonary-Critical Care Medicine Branch, National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20892-1590

The dimer of integrin $\alpha7$ and $\beta1$ is a major laminin-binding receptor in skeletal muscle. We studied interactions of integrin $\alpha7\beta1$ with the extracellular matrix protein laminin in solution and in intact cells. Integrin $\alpha7\beta1$ bound to EHS laminin (laminin-1, composed of $\alpha1$, $\beta1$, and $\gamma1$ chains), but not to endogenous laminin expressed in C2C12 myotubes. Northern blot analysis demonstrated that C2C12 myotubes synthesized laminin-1 α, β, and γ subunits mRNAs. C2C12 laminin was, however, immunologically distinct from EHS laminin; it was not recognized by 5D3 anti-laminin-1 monoclonal antibody, whereas 5A2 and LT3 antibodies reacted equally well with C2C12 and EHS laminins. Following deglycosylation of EHS laminin, separation of the subunits by SDS–PAGE, Western blotting, and partial amino acid sequencing of the protein bands, the epitope recognized by 5D3 antibody was localized to the $\gamma1$ laminin chain. Following binding *in vitro,* the complex of EHS laminin and integrin $\alpha7\beta1$ was subject to chemical cross-linking. The two proteins did not undergo cross-linking at the cell surface, consistent with the fact that in intact, resting myotubes integrin $\alpha7\beta1$ interacted poorly with EHS laminin, which may reflect a limited accessibility of integrin $\alpha7\beta1$ in the membrane to laminin or an inactive state of the integrin.
© 1998 Academic Press

INTRODUCTION

Integrins are a family of heterodimeric cell surface receptors which are involved in cell-extracellular matrix (ECM) and cell–cell interactions [1–3]. In adult, normal tissues, integrin $\alpha7$ is most abundant in skeletal and cardiac muscle [4], where, as a dimer with integrin $\beta1$, it mediates interactions with the ECM protein laminin [5]. Early during development, integrin $\alpha7$ is expressed in nervous system and in myotome [6]. In adult skeletal muscle, integrin $\alpha7$ is concentrated in the myotendinous and neuromuscular junctions [7]. It can exist in one of several different isoforms that result from alternative mRNA splicing [8–10], with each splice variant showing a distinct spatiotemporal pattern of expression. It has been postulated that, together with $\beta1$, integrin $\alpha7$ plays a crucial role in myoblast adhesion and migration on laminin substrate [11] and, eventually, in the process of muscle development and repair. Nonmyogenic cells, which did not migrate on laminin and lacked endogenous integrin $\alpha7$, but which were $\beta1$-positive, exhibit increased adhesion [12, 13] or motility [12, 14] on laminin after transfection with the integrin $\alpha7$ gene.

Laminins are heterotrimeric extracellular matrix proteins composed of α, β, and γ chains [15, 16]. They play an important role in the growth, locomotion, and differentiation of many cell types, including myoblasts [17–20]. Both myoblasts and differentiated myotubes synthesize laminin and deposit it at the cell surface [17, 21, 22], although the expression of the individual laminin subunits is regulated differently during myogenic differentiation. Laminin-1 ($\alpha1$-$\beta1$-$\gamma1$), which is downregulated during differentiation, or laminin-2 ($\alpha2$-$\beta1$-$\gamma1$, merosin) and laminin-4 ($\alpha2$-$\beta2$-$\gamma1$, *s*-merosin), which are upregulated, are required for myoblast fusion, but only merosins promote myotube stability by preventing apoptosis [23]. The effects of laminins on intracellular signaling pathways are mediated by integrin and nonintegrin receptors, including α-dystroglycan [24]. Molecular heterogeneity of laminins provides the means for regional specialization of ECM. For example, in the basal lamina that ensheathes skeletal muscle fibers, $\alpha1$ and $\beta2$ subunits of laminin are specifically concentrated in the synaptic area, $\beta1$ chain is excluded from the synapse, and several other subunits are distributed equally between the synaptic and extrasynaptic portion of the lamina [25, 26].

Integrin $\alpha7$ is a major substrate for a cell-surface, glycosyl-phosphatidylinositol-anchored ADP-ribosyltransferase [27, 28] in intact C2C12 myotubes [29]. In the presence of micromolar concentrations of extracellular NAD, an arginine located in the 39-kDa domain of integrin $\alpha7$, between amino acids 575 and 886, is ADP-ribosylated [29]. The ADP-ribose is cleaved by an

[1] Current address: Department of Biochemistry, Kansas State University, Manhattan, KS 66506.
[2] To whom correspondence and reprint requests should be addressed. Fax: (301) 496 2363. E-mail: mossj@fido.nhlbi.nih.gov.

0014-4827/98 $25.00
Copyright © 1998 by Academic Press
All rights of reproduction in any form reserved.

extracellular 5′-nucleotide phosphodiesterase, generating a phosphoribose moiety in the integrin structure [30]. The role of this posttranslational modification in integrin function is not clear. To further understand the function of integrin α7, we studied the interaction of α7β1 dimer with laminin-1, both *in vitro* and in intact myotubes. The ability of integrin α7β1 to bind laminin-1 was found to correlate with the presence of a specific isoform containing an epitope recognized by 5D3 anti-laminin-1 antibody, but not with epitopes recognized by other anti-laminin-1 antibodies. Further, binding of integrin α7β1 to laminin-1 in solution, after extraction from the membrane with detergent, clearly differed from that in the context of an intact cell, suggesting that integrin α7β1 in myotubes might have been masked or inactive.

EXPERIMENTAL PROCEDURES

Cell culture. C2C12 mouse myoblasts (American Tissue Culture Collection, Rockville, MD) were grown on plastic dishes in Dulbecco's modified Earle's medium with 10% fetal bovine serum under a humidified atmosphere with 5% CO_2. Cells were plated at a density $\sim 10^3/cm^2$, reached confluency on day 4, and were allowed to grow for 6–8 more days until numerous well-differentiated myotubes were present. In some experiments, integrin α7 was [^{32}P]ADP-ribosylated at the surface of intact cells by incubation for 30 min at 37°C in 2 ml of DPBS with 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1 mM ADP-ribose, and 5 μM [adenylate-^{32}P]NAD (4 Ci/mmol).

Laminin binding. To study binding in solution of laminin to integrin α7β1, cells on each 100-mm plate were treated for 10 min with 2 ml of lysis buffer (20 mM Tris · Cl (pH 7.5), 150 mM NaCl, 50 mM octyl glucoside, 1 mM $MnCl_2$, 1 mM AEBSF, 5 μg/ml each of leupeptin and aprotinin) and pelleted by centrifugation (16,000*g*, 20 min, 4°C). The supernatant (1 ml) was incubated for 30 min with EHS mouse laminin (Life Technologies, Gaithersburg, MD) on ice, at indicated concentrations. To study binding on intact cells, myotubes were incubated for 30 min at 37°C in Puck's saline (182 mM NaCl, 5.4 mM KCl, 4 mM $NaHCO_3$, 5.5 mM glucose) containing laminin at the indicated concentration and 1 mM $MnCl_2$, unless indicated otherwise. Phosphate-based buffer was omitted to avoid precipitation of phosphate · manganese complexes. Similar results were obtained when myotubes were incubated in DPBS[3] with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$.

Immunoprecipitation and detection of laminin and integrin. Cells on each 100-mm plate were treated with 2 ml of lysis buffer, scraped, and centrifuged (16,000*g*, 20 min, 4°C). For immunoprecipitation of laminin, the supernatant was incubated for 2 h on ice with rat anti-mouse laminin monoclonal antibodies 5A2, 5C1, or 5D3 (5 μg/ml; Life Technologies, Gaithersburg, MD, or Dr. Dale R. Abrahamson, University of Alabama, Birmingham, AL), then for 1 h with rabbit anti-rat IgG (5 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), followed by 30 min with protein G–Sepharose (50 μl bed volume; Pharmacia, Sweden). Sepharose beads were washed four times with 1 ml of lysis buffer. Laminin was released from the beads with 50 μl of SDS–PAGE gel-loading buffer. In some experiments, integrin α7β1, which coprecipitated with laminin, was released by washing the beads with the lysis buffer containing 10 mM EDTA instead of $MnCl_2$, before incubation with SDS. For immunoprecipitation of integrin α7, the supernatant from cells on one 100-mm plate was incubated with a rabbit antibody raised against a peptide from the cytoplasmic domain (splice variant B) of integrin α7 (1:250 dilution, a gift from Dr. Stephen J. Kaufman, University of Illinois, Urbana, IL), and then with protein G–Sepharose. Individual laminin subunits were resolved by SDS–PAGE under reducing conditions, transferred to nitrocellulose membranes, incubated with LT3 (Upstate Biotechnology, Inc.), 5A2, or 5D3 antibodies (each at 5 μg/ml), followed by anti-rat IgG-horseradish peroxidase conjugate, and visualized by an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Integrin α7 was detected in SDS gels by silver staining, or, if [^{32}P]ADP-ribosylated, by autoradiography.

Northern blotting. mRNA was isolated from C2C12 myotubes using a FastTrack 2.0 kit (Invitrogen, San Diego, CA); RNA (5 μg) was subjected to electrophoresis in a denaturing 0.8% agarose gel containing formaldehyde and transferred to a nylon membrane. Prehybridization, hybridization, and radiolabeling of oligoprobes were performed as described [25]. The following oligoprobes, specific for the laminin α1 subunit, were used: CACTGTACTGCCCGGCAGTGTTCAGGCTTCTCCTCTCCTTTGCAACAC or TCAGCTGGTCACGGTCAATCTCCCTTCTGGTATTAAAGTCCCGGAAGT (inverse complement of nucleotides 1091–1138 and 2000–2047, respectively); specific for the laminin β1 subunit: TCAAAGTGACACGAGCTGGAATGTTCATTGCAGTTACATTTTTTGCAG, AACCTGGGCCTTTTCTGCTTCTTCTAATGCTTCCTTCACCATGTCTGC, or TAAGCAGGTGCTGTAAACCGCAACTTTCTCACTTATGTCCTTAAGGAG (inverse complement of nucleotides 1170–1217, 4951–4998, or 5488–5535 respectively); specific for the laminin γ1 subunit: TTGTCAAAGTTGTAGGCACTCGGCCGTCCTTCCAGGGTTGAAAAGGCC, CAGCGCTAGCTGGGCCTCCCTTGTCTTCTCATTGGCTTCAGCTATGGT, or CTTCTCGATAGACGGGGTGTTGAAGCAGCCGGTTGGTAGGGTCTTCTT (inverse complement of nucleotides 846–893, 4369–4416, or 4957–5004, respectively).

De-N-glycosylation and amino acid sequencing of EHS laminin subunits. EHS laminin (0.7 mg/ml, 200 μl total volume) was incubated for 2 min at 90°C in 20 mM Tris · Cl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 0.1% SDS, and 0.5% β-mercaptoethanol, unless indicated otherwise. Then 0.5% octyl glucoside, endoglycosidase-F (40 units; Oxford Glycosystems, Rosedale, NY), and peptide-*N*-glycosidase F (40 units; Oxford Glycosystems) were added and incubation was continued for 16 h at 37°C. Individual laminin subunits were resolved by SDS–PAGE in 4% gels under reducing conditions and stained with Coomassie blue or transferred to nitrocellulose membranes for reaction with different anti-laminin antibodies. Coomassie blue-stained bands of ~210 and 190 kDa, which were observed after deglycosylation of the denatured laminin and corresponded to immunoreactive species, were excised from the gel and analyzed by Lys-C endoprotease digestion, separation of peptides by reversed-phase HPLC, and amino acid sequencing (Dr. William Lane, Harvard Microchemistry Facility, Cambridge, MA).

Protein cross-linking. Protein complexes were cross-linked with DTSSP (Pierce, Rockford, IL), a membrane-impermeant, thiol-cleavable cross-linker. Since DTSSP reacted with primary amines, cross-linking was performed in buffers lacking these functional groups. For cross-linking solubilized proteins, myotubes on each 100-mm plate were treated with lysis buffer (2 ml) containing 20 mM Hepes · KOH, pH 7.5, instead of Tris · Cl, and 1 mM PMSF instead of AEBSF, and centrifuged. The supernatant (1 ml) was incubated with indicated amounts of laminin for 30 min on ice before addition

---

[3] Abbreviations used: EHS laminin, laminin isolated from Engelbreth–Holm–Swarm tumor; DPBS, Dulbecco's modified phosphate-buffered saline; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; AEBSF, 4-(2-Aminoethyl)-benzenesulfonyl fluoride; PMSF, Phenylmethylsulfonyl fluoride; DTSSP, 3,3′-dithiobispropionimidate.

[4] Since antibodies specific for mouse laminin α1 or α2 were not available to us, only expression of laminin subunit mRNA could be evaluated.

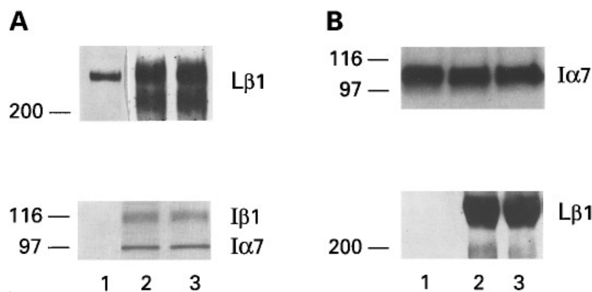

FIG. 1. Formation of a stable complex of integrin $\alpha 7\beta 1$ and EHS laminin in solution. C2C12 myotube lysate (1 ml) was incubated with EHS laminin (10 μg; lanes 2 and 3) or without laminin (lane 1) and immunoprecipitated with 5A2 anti-laminin $\beta 1$ antibody (A) or anti-integrin $\alpha 7$ antibody (B). (A) The amount of immunoprecipitated laminin $\beta 1$ was assessed by Western blotting with 5A2 antibody. Integrin $\alpha 7\beta 1$ bound to laminin was eluted from the immunoprecipitate with buffer containing EDTA, and analyzed by SDS–PAGE and silver staining. (B) Cells were incubated with [^{32}P]NAD prior to lysis, EHS laminin binding, and immunoprecipitation. The amount of [^{32}P]ADP-ribosylated and immunoprecipitated integrin $\alpha 7$ was estimated after SDS–PAGE and autoradiography. Laminin, which coprecipitated with integrin $\alpha 7$, was detected by Western blotting using 5A2 antibody. In lane 3, the immunoprecipitate was subjected to an additional 3 h wash before SDS–PAGE. Positions of molecular weight markers (kDa) are indicated on the left, laminin and integrin subunits on the right. Abbreviations are: I$\alpha 7$, integrin $\alpha 7$; I$\beta 1$, integrin $\beta 1$; L$\beta 1$, laminin $\beta 1$.

of 0.5 mM DTSSP. After 30 min, cross-linking was stopped by adding 50 mM Tris · Cl, pH 7.5, and proteins were immunoprecipitated. For cross-linking laminin to cell-surface proteins in laminin overlay experiments, myotubes were incubated for 30 min at 37°C in Puck's saline (2 ml) containing 1 mM $MnCl_2$ and laminin, and then for next 30 min with 0.5 mM DTSSP, washed, and lysed with 2 ml of Tris · Cl-containing lysis buffer. EHS laminin, which was supplied in Tris · Cl buffer, was dialyzed against Puck's saline prior to cross-linking. In another set of experiments, cells were detached from plates with DPBS containing 5 mM EDTA, washed with Puck's saline containing 1 mM $MnCl_2$, and plated on laminin-coated dishes. After 1 h, when most of the cells had adhered to plates, 0.5 mM DTSSP was added and incubation was continued for 30 min, followed by a wash and lysis with Tris · Cl-containing lysis buffer. Similar results were obtained when myotubes were incubated in DPBS with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ instead of Puck's saline. When [^{32}]ADP-ribosylation of integrin $\alpha 7$ was performed first, the cross-linking buffer contained 1 mM IDP instead of ADP-ribose, routinely used to inhibit removal of the radiolabel from the integrin by a 5′-nucleotide phosphodiesterase [30].

RESULTS

Integrin $\alpha 7\beta 1$ interacts *in vitro* with laminin purified from mouse Englebreth–Holm–Swarm (EHS) tumor (laminin-1, composed of $\alpha 1$, $\beta 1$, and $\gamma 1$ chains) and it can be efficiently purified on an EHS laminin affinity column [4, 5, 29]. Here, we studied the interaction between these two proteins by immunoprecipitation with anti-laminin or anti-integrin antibody. When EHS laminin was added to C2C12 mouse myotube lysate, a complex of integrin $\alpha 7\beta 1$ and laminin was formed. Integrin $\alpha 7\beta 1$ was detected in the immunoprecipitate obtained with an anti-laminin antibody (Fig. 1A, lane 2), and laminin was present in the material immunoprecipitated with an anti-integrin $\alpha 7$ antibody (Fig. 1B, lane 2). Results were similar when 5A2, 5C1, or 5D3 anti-laminin antibodies were used for immunoprecipitation. The complex of integrin $\alpha 7\beta 1$ and EHS laminin seemed to be relatively stable, since the amounts of the proteins were unchanged after incubation of the precipitate for 3 h in washing buffer (Figs. 1A and 1B, lane 3).

Skeletal muscle myoblasts synthesize laminin [17, 21–23] and, consistently, laminin $\beta 1$ chain was detected by Western blotting in the immunoprecipitate from C2C12 myotubes (Fig. 1A, lane 1). Integrin $\alpha 7\beta 1$ did not, however, coimmunoprecipitate with endogenous laminin, nor was endogenous laminin isolated with anti-integrin $\alpha 7$ antibody (Fig. 1B, lane 1). When equivalent amounts of myotube and EHS laminins were isolated by immunoprecipitation, washed with buffer containing EDTA and lactose to remove lectin L-14 and other proteins possibly bound to the endogenous laminin that could block subsequent integrin binding [31, 32], and mixed with C2C12 cell lysate, integrin $\alpha 7$ was retained only by EHS laminin and not by the myotube laminin (Fig. 2).

It had been observed that the expression of laminin $\alpha 1$ chain was downregulated and $\alpha 2$ subunit upregulated during myogenic differentiation [23]. It seemed

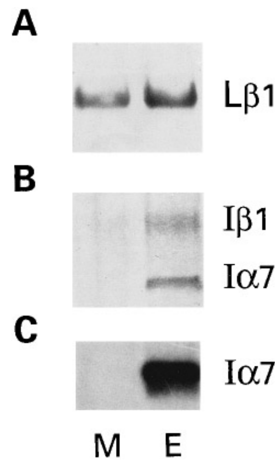

FIG. 2. Binding of integrin $\alpha 7\beta 1$ to EHS laminin and to endogenous laminin produced by C2C12 myotubes. Endogenous laminin produced by C2C12 myotubes on one 100-mm plate (M) or an equivalent amount of EHS laminin (0.7 μg, E) was immunoprecipitated with 5A2 antibody and washed with buffer containing 10 mM EDTA and 0.1 M lactose. On a parallel plate, C2C12 myotubes were incubated with [^{32}P]NAD, lysed, and mixed with each laminin immunoprecipitate. Integrin $\alpha 7\beta 1$ retained on laminin was then eluted with buffer containing EDTA and analyzed by SDS–PAGE and silver staining (B) or autoradiography (C). The amount of laminin in the immunoprecipitate, after treatment with SDS sample buffer, was estimated by SDS–PAGE and Western blotting with 5A2 antibody (A). Abbreviations are as in Fig. 1.

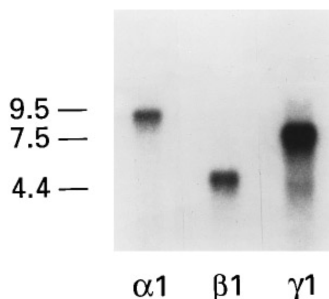

FIG. 3. Northern analysis of C2C12 myotube RNA. mRNA from C2C12 myotubes (5 μg) was hybridized with oligoprobes specific for laminin $\alpha1$, $\beta1$, and $\gamma1$ subunits, as indicated. Positions of molecular weight markers (kb) are on the left.

probable, therefore, that the composition of laminin subunits produced by C2C12 myotubes differed from those of EHS laminin, accounting for the apparently different affinities of integrin $\alpha7\beta1$ for the two proteins. Northern blot analysis of C2C12 RNA revealed, however, that mRNA's for all laminin-1 subunits, i.e., $\alpha1$, $\beta1$, and $\gamma1$, were present in the myotubes (Fig. 3). In fact, using several different oligoprobes, it was found that the level of laminin $\alpha2$ mRNA was significantly lower than $\alpha1$ mRNA (result not shown), which can be attributed to the continuous presence of high serum concentrations in the myoblast growth medium [23]. Therefore, it seems conceivable that, in C2C12 myotubes, laminin $\alpha1$, $\beta1$, and $\gamma1$ subunits were synthesized and assembled into type 1 laminin.

EHS and myotube laminins were distinguished by their reactivity with several different anti-laminin antibodies. Amounts of C2C12 and EHS laminin that were equivalent by Western blotting with 5A2 and LT3 antibodies, differed dramatically in their reactivity with 5D3 antibody. C2C12 laminin was not recognized by 5D3 antibody, whereas EHS laminin reacted well (Fig. 4).

5A2 and 5D3 antibodies were originally raised against mouse EHS laminin, and the domains recognized by those antibodies were established by rotary shadow electron microscopy [33]. 5A2 antibody bound to a site on one of the short arms of laminin, which was later demonstrated to be the $\beta1$ chain [26]. 5D3 antibody site was localized to the long arm of laminin, comprising all three laminin subunits, ~14 nm from the terminal globular domain, within the E8 fragment generated by elastase digestion [33]. It had not been determined which laminin subunit within the long arm reacted with antibody 5D3. Since 5D3 was the only antibody capable of distinguishing between EHS and C2C12 laminins in our experiments, we identified the laminin chain containing 5D3 epitope. When EHS laminin was subjected to SDS–PAGE under reducing conditions, two protein bands of ~400 kDa ($\alpha1$ subunit) and ~220 kDa (unresolved $\beta1$ and $\gamma1$ subunits, reactive with all three antibodies) were observed (Fig. 5, lane 1). Following enzymatic de-N-glycosylation, three distinct laminin bands, which represented individual laminin subunits, were observed on gels (Fig. 5, lane 3). Thermal denaturation and reduction of EHS laminin were required for effective de-glycosylation and resolution of $\beta1$ and $\gamma1$ chains (Fig. 5, compare lanes 2 and 3). The ~210-kDa band reacted with 5A2 antibody and was confirmed by microsequencing to be the $\beta1$ subunit. Two peptides were separated from this protein after cleavage with Lys-C protease. Their amino acid sequences, NSDIQGALDSITK and DILAQSPAAEPLK, correspond to residues 1311–1232 and 1232–1244, respectively, in the mouse laminin $\beta1$ sequence. The ~190-kDa species reacted with antibodies LT3 and 5D3 and was identified as the $\gamma1$ chain. Sequences of two peptides derived from this protein, GRSTLQEANDILNNLK and EGFFGNPLAPNPADK, represent residues 1352–1367 and 864–878 in the mouse laminin $\gamma1$ subunit. RT-PCR analysis of the $\gamma1$ mRNA (residues 4177–5007, corresponding to amino acids 1329–1605, contained within the E8 fragment of laminin) did not reveal any evidence of alternative splicing of $\gamma1$ mRNA in C2C12 cells; only one amino acid difference was observed (Arg at position 1473 for Lys).

Since EHS laminin bound well to integrin $\alpha7\beta1$ *in vitro,* we investigated the interactions between those two proteins in the context of an intact cell. Myotubes were incubated with EHS laminin, washed, lysed, and subjected to immunoprecipitation with anti-laminin antibody. Total amount of laminin associated with cells was estimated based on the blotting of known amounts of EHS laminin (Fig. 6A). Amounts of integrin $\alpha7$ found in the immunoprecipitates were dependent on the laminin concentration during incubation with intact cells

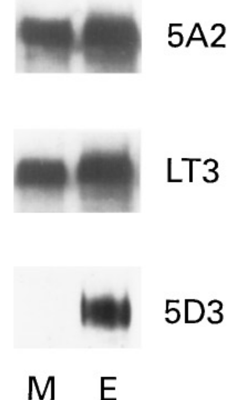

FIG. 4. Immunological distinction between C2C12 laminin and EHS laminin. C2C12 myotube lysate (20 μl, corresponding to 1% of cells on a 100-mm plate; M) or EHS laminin (0.2 μg; E) was analyzed by Western blotting with anti-laminin antibodies 5A2, LT3, or 5D3, as indicated.

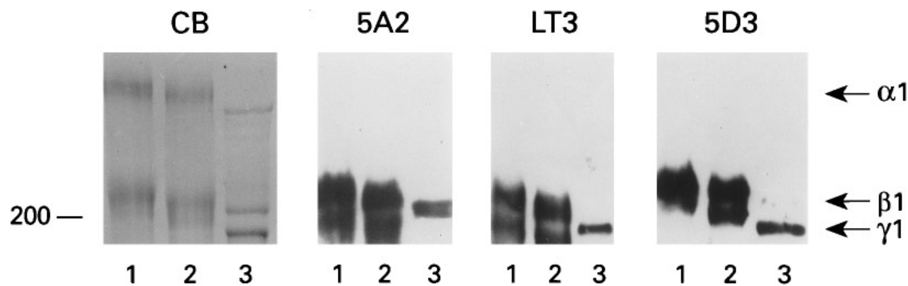

FIG. 5. Localization of epitopes recognized by 5A2, LT3, and 5D3 antibodies to individual EHS laminin subunits. EHS laminin (lane 1) was treated with Endoglycosidase F and Peptide-*N*-glycosidase F (60 units of each enzyme/mg of laminin) for 16 h at 37°C in its native form (lane 2) or after denaturation with 0.1% SDS and 0.5% β-mercaptoethanol and heating to 90°C, as described under Experimental Procedures (lane 3). Coomasie blue (CB)-stained SDS–polyacrylamide gel and Western blots with antibodies 5A2, LT3, or 5D3 are shown. Two separate protein bands of ~200-kDa, generated by the deglycosylation of denatured EHS laminin (A, lane 3), were confirmed by microsequencing to be laminin $\beta1$ and $\gamma1$ subunits, as indicated. The third distinct protein band of a significantly higher molecular weight was assumed to be the laminin $\alpha1$ subunit.

(Fig. 6B, closed triangles). The relationship between integrin $\alpha7$ and EHS laminin retained by cells and present during the immunoprecipitation (Fig. 6B, open triangles) was, however, identical to the relationship observed when the interaction occurred in solution (Fig. 6B, closed circles). Since the complex of integrin $\alpha7\beta1$ and EHS laminin was stable over a period of several hours (Fig. 1, lane 3), one would expect larger amounts of integrin $\alpha7$ being isolated if the complex was formed in intact cells, when the actual concentration of laminin was higher. In our studies, EHS laminin could have been retained at the surface of intact myotubes by a different receptor or even nonspecifically, followed by interaction with integrin later on, in solution, after lysis of the cells.

Binding of EHS laminin to integrin $\alpha7\beta1$ required Mn^{2+} ions and did not occur in the presence of Ca^{2+} and Mg^{2+} ions (Table 1). When intact myotubes were incubated with EHS laminin in the presence of either Mn^{2+} or Ca^{2+} and Mg^{2+}, then washed, lysed in buffer containing Mn^{2+}, and subjected to immunoprecipitation with anti-laminin antibody, the amounts of integrin $\alpha7$ co-precipitated with laminin were the same (Table 1). This is consistent, again, with formation of the integrin $\alpha7\beta1 \cdot$ EHS complex laminin after cell lysis, in the presence of manganese, rather than at the cell surface.

To address directly the question of the interaction of EHS laminin with integrin $\alpha7\beta1$ in intact myotubes, chemical cross-linking of the laminin · integrin complex was performed. The cross-linker,

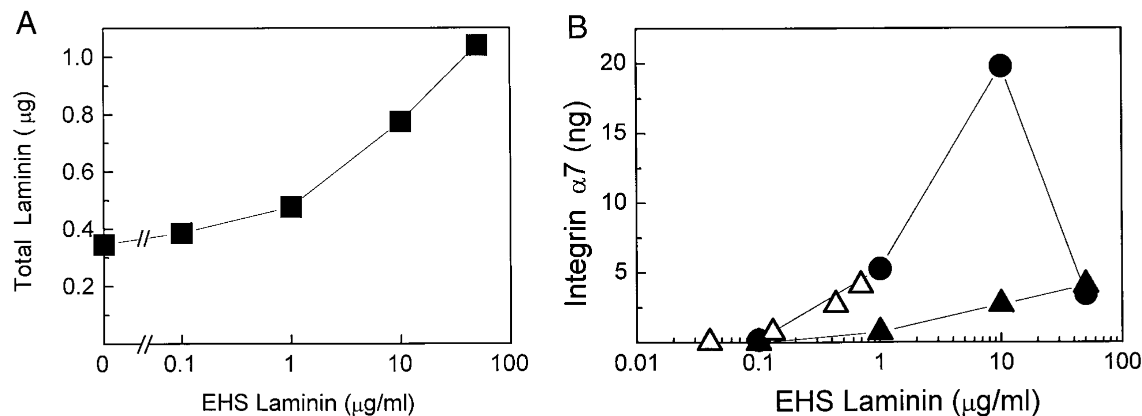

FIG. 6. Binding of EHS laminin to C2C12 myotubes. Intact C2C12 myotubes were incubated in Puck's saline with 1 mM $MnCl_2$ for 30 min at 37°C in the presence of EHS laminin at indicated concentrations, washed, lysed, and either analyzed by SDS–PAGE and Western blotting using LT3 antibody (A) or subjected to immunoprecipitation with 5A2 antibody (B). (A) Total amounts of laminin associated with cells on one 100-mm plate were estimated by comparing intensities of laminin bands on Western blots with intensities corresponding to known amounts of EHS laminin. (B) The amount of integrin $\alpha7$ bound to laminin was quantified after incubation of the immunoprecipitate with buffer containing EDTA, gel electrophoresis of the eluate, and silver-staining. Integrin $\alpha7$ is plotted as a function of EHS laminin concentration present during incubations with C2C12 cells (▲) or EHS laminin bound to cells, as estimated from A (△). Alternatively, EHS laminin was added to lysed cells, laminin · integrin $\alpha7\beta1$ complex formed in solution was immunoprecipitated, and the amount of integrin $\alpha7$ in the precipitate was plotted as a function of laminin concentration present during immunoprecipitation (●).

TABLE 1

Effects of Divalent Cations on Formation of EHS Laminin Integrin $\alpha7\beta1$ Complex

	Mn^{2+}	Ca^{2+}/Mg^{2+}
In vitro		
Integrin $\alpha7$ (ng)	20 ± 4	0
Laminin $\gamma1$ (ng)	390 ± 50	420 ± 40
In intact myotubes		
Integrin $\alpha7$ (ng)	3.1 ± 0.4	2.6 ± 0.4
Laminin $\gamma1$ (ng)	80 ± 9	78 ± 7

Note. C2C12 myotubes from one 100-mm plate were lysed with buffer containing either 1 mM $MnCl_2$ or 0.5 mM each of $CaCl_2$ and $MgCl_2$ and incubated with EHS laminin (10 μg/ml). Alternatively, intact myotubes were incubated with EHS laminin (10 μg/ml) in the presence of 1 mM $MnCl_2$ or 0.5 mM each of $CaCl_2$ and $MgCl_2$, washed, and treated with lysis buffer containing 1 mM $MnCl_2$. Laminin · integrin $\alpha7\beta1$ complex was immunoprecipitated with 5A2 anti-laminin antibody. Integrin $\alpha7$ was eluted with a buffer containing EDTA, subjected to SDS–PAGE, and silver stained. Laminin was eluted with SDS sample buffer and analyzed by SDS–PAGE and Western blotting with LT3 antibody. Intensities of integrin or laminin bands were quantified by densitometry and compared with known amounts of a standard protein or laminin, respectively. Data are the means ± SE from three experiments.

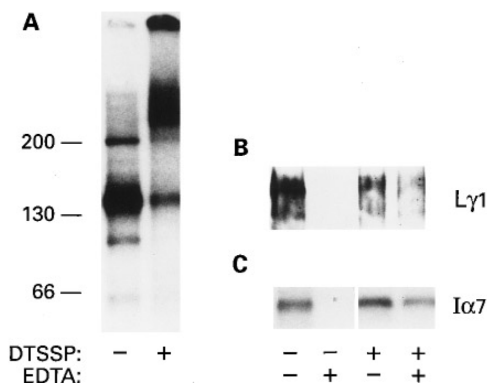

FIG. 7. Cross-linking of laminin and integrin $\alpha7$ in solution. C2C12 myotubes on each 100-mm plate were incubated with [^{32}P]-NAD, washed, treated with lysis buffer (2 ml), and centrifuged, as described under Experimental Procedures. The supernatant (1 ml) was incubated for 30 min at 4°C with EHS laminin (10 μg/ml) and treated with 1 mM DTSSP. Laminin · integrin $\alpha7$ complex was immunoprecipitated with anti-integrin $\alpha7$ (B) or 5A2 anti-laminin antibody (C) and washed with buffer without or with 10 mM EDTA, as indicated. (A) Radiolabeled integrin $\alpha7$ before and after DTSSP treatment was analyzed by nonreducing SDS–PAGE and autoradiography. (B) Laminin, coprecipitated with $\alpha7$, was detected by Western blotting with LT3 antibody. (C) Radiolabeled integrin $\alpha7$, coprecipitated with laminin, was detected by autoradiography.

DTSSP, contained a disulfide bond, which allowed dissociation of the cross-linked proteins under reducing conditions. First, cross-linking of the laminin · integrin complex was performed in solution. As demonstrated by the position of the radiolabeled integrin $\alpha7$ on SDS–PAGE without reduction, high molecular weight, cross-linked structures were formed (Fig. 7A). Following incubation with EHS laminin, the laminin · integrin complex was immunoprecipitated with either anti-integrin $\alpha7$ antibody (Fig. 7B) or anti-laminin antibody (Fig. 7C). Washing the immunoprecipitate with EDTA completely disrupted the complex, unless cross-linking had been performed first. When the cross-linker was applied to intact C2C12 myotubes incubated with EHS laminin, notably different results were obtained. In this case, there was no immunoprecipitation of laminin with anti-integrin antibody (Fig. 8B) or integrin with anti-laminin antibody (Fig. 8C) after the EDTA wash. Integrin $\alpha7$ became covalently attached, however, to other, unidentified proteins at the cell surface, as manifested by its migration in a nonreducing gel (Fig. 8A). Therefore, in intact myotubes, EHS laminin did not seem to be complexed with integrin $\alpha7\beta1$.

To exclude the possibility that integrin $\alpha7\beta1$ in intact cells binds only a multivalent ligand and not its soluble form, myotubes were detached from culture plates with medium containing EDTA, washed, plated on dishes coated with EHS, and incubated with the cross-linker before immunoprecipitation. Again, washing of the immunoprecipitate with EDTA resulted in complete dissociation of the integrin · laminin complex (Figs. 9A and 9B), irrespective of cross-linking. In this experiment, cross-linking itself interfered with the coimmunoprecipitation of the two proteins, although not with recognition by the antibodies. The amounts of integrin isolated with anti-integrin antibody (Fig. 9A) and laminin isolated

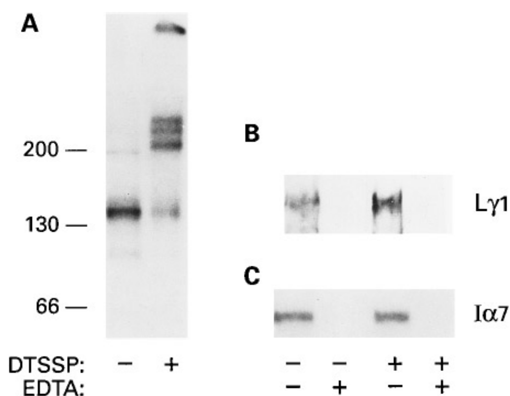

FIG. 8. Inability to cross-link soluble EHS laminin and integrin $\alpha7$ at the surface of C2C12 myotubes. C2C12 myotubes were incubated with [^{32}P]NAD, washed, and incubated with EHS laminin (10 μg/ml) in Puck's saline (2 ml) with 1 mM $MnCl_2$ for 30 min before addition of 1 mM DTSSP, cell lysis, and immunoprecipitation. The extent of cell surface cross-linking of integrin $\alpha7$, as well as formation of the laminin · integrin complex was examined in A, B, and C, respectively, as described in the legend to Fig. 7.

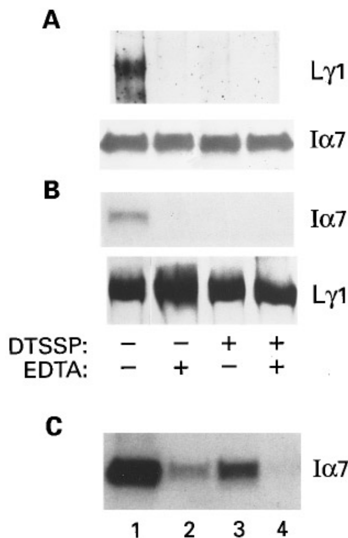

FIG. 9. Inability to cross-link immobilized EHS laminin to integrin $\alpha7$ at the surface of C2C12 cells. C2C12 myotubes were incubated with [^{32}P]NAD, detached from plates with 5 mM EDTA, washed, suspended in buffer containing 1 mM $MnCl_2$, and plated on a laminin-coated dish. After 30 min, 1 mM DTSSP was added to adherent cells. Laminin·integrin $\alpha7$ complex was immunoprecipitated with anti-integrin $\alpha7$ antibody (A) or 5A2 anti-laminin antibody (B) and washed with buffer without or with 10 mM EDTA, as indicated. (A) Laminin coprecipitated with $\alpha7$ was detected with LT3 antibody. Treatment of cells with DTSSP or washing of the immunoprecipitate with EDTA did not alter the amount of immunoprecipitated, radiolabeled integrin $\alpha7$, as documented by autoradiography. (B) Integrin $\alpha7$, coprecipitated with laminin, was detected by autoradiography. Immunoprecipitation of laminin was not affected by DTSSP treatment of cells or washing of the immunoprecipitate with EDTA, as verified by Western blotting with LT3 antibody. (C) C2C12 myotubes were detached, plated on EHS laminin-coated dishes, and incubated without (lanes 1 and 2) or with DTSSP (lanes 3 and 4), followed by laminin immunoprecipitation. On a parallel plate, myotubes were treated for 30 min with [^{32}P]NAD, washed, and then further incubated without (lanes 1 and 3) or with DTSSP (lanes 2 and 4). Radiolabeled myotubes were lysed and mixed with laminin immunoprecipitates. Binding of [^{32}P]ADP-ribosylated integrin $\alpha7$ to laminin was detected by SDS–PAGE and autoradiography. Exposure of either integrin $\alpha7$ (lane 2) or laminin (lane 3) or both proteins (lane 4) to DTSSP in intact myotubes decreased the extent of binding.

with anti-laminin antibody (Fig. 9B) were similar with or without cross-linking, in the presence or absence of EDTA. The lack of coimmunoprecipitation of laminin and integrin observed after addition of cross-linker (Figs. 9A and 9B, lane 2) is consistent with the notion that the interaction between those two proteins did not occur at the cell surface, prior to cross-linking, but rather in solution, after lysis of the cells. Since cross-linking after cell detachment and adhesion (Fig. 9) involved much lower quantities of laminin than did the experiment with soluble laminin (Fig. 8), it is possible that it resulted in a more complete modification of laminin, which was then unable to interact with integrin in solution. Indeed, exposure of laminin (Fig. 9C, lane 3), integrin (Fig. 9C, lane 2), or both (Fig. 9C, lane 4), to the cross-linker decreased the extent of subsequent interaction of the two proteins.

DISCUSSION

Integrin $\alpha7\beta1$ is considered a major laminin-binding integrin in skeletal muscle. We studied interactions between laminin and integrin $\alpha7\beta1$ both *in vitro* and in intact myotubes by immunoprecipitating complexes of the two proteins with anti-laminin or anti-integrin antibodies. We show that, whereas integrin $\alpha7\beta1$ binds EHS laminin well *in vitro,* its interaction with the same ligand, either monovalent or multivalent, did not occur in intact cells. Laminin produced by C2C12 myotubes does not form a complex with integrin $\alpha7\beta1$ either *in vitro* or in cells.

Anti-integrin antibody used in our studies reacted only with $\alpha7$B splice variant. Since differentiated myotubes express both $\alpha7$B and $\alpha7$A forms, with different splicing in their cytoplasmic domains [8–10], those experiments provided information about only a subpopulation of integrin $\alpha7$ molecules present in the myotubes. The results obtained with anti-integrin antibody paralleled, however, the findings obtained with anti-laminin antibodies (Figs. 1 and 7–9), which may reflect similar ligand binding properties of integrin $\alpha7$A and $\alpha7$B isoforms.

Of the several anti-laminin monoclonal antibodies used, 5D3 antibody was found particularly useful, since the presence of an epitope recognized by this antibody correlated with laminin ability to bind to integrin $\alpha7\beta1$. 5D3 antibody did not interfere, however, with the formation of laminin·integrin complex, suggesting that binding sites for integrin $\alpha7\beta1$ and the antibody were different and not located in close proximity. Rather, the 5D3 epitope could have more global structural effects on the laminin molecule that affect the integrin $\alpha7\beta1$ binding site. This is consistent with the fact that both integrin $\alpha7\beta1$ [5] and 5D3 antibody [33] bound the E8 fragment of laminin, generated by elastase digestion.

The epitope recognized by 5D3 antibody had been localized by rotary shadow electron microscopy to the long arm of laminin, ~14 nm from the terminal globular domain [33], within the rod-like structure formed by a triple coiled-coil helix and comprising $\alpha1$, $\beta1$, and $\gamma1$ chains [34]. Here, we mapped the recognition site of 5D3 to the $\gamma1$ chain of EHS laminin. This is consistent with different patterns of immunostaining by 5D3 and 5A2 antibodies in the renal glomerular basement membrane [35]. Those two antibodies react with a ~220-kDa protein band in Western blots following SDS–PAGE under reducing conditions, which contains unresolved $\beta1$ and $\gamma1$ laminin chains. 5A2 antibody

was unequivocally shown to recognize $\beta1$ chain by staining fibroblasts transfected with a laminin $\beta1$ expression vector [26]. Here, following de-N-glycosylation, we were able to resolve and identify by microsequencing $\beta1$ and $\gamma1$ laminin subunits, which indeed reacted differently with 5A2 and 5D3 antibodies.

De-glycosylated laminin $\gamma1$, but not the $\beta1$ chain, also reacted with LT3 antibody. This is incompatible with the notion that LT3 antibody recognizes mouse laminin $\beta1$ subunit, which was established previously by comparative immunofluorescence on sections of human tissues [36].

The reason that 5D3 antibody reacted differently with EHS laminin and laminin synthesized by C2C12 myotubes is not clear. C2C12 mRNA hybridized with several $\gamma1$-specific oligoprobes, and $\gamma1$ protein was detected with LT3 antibody in the immunoprecipitates from C2C12 cells obtained with 5A2 antibody (Fig. 9). Therefore, it appears that C2C12 myotubes were capable of synthesizing the $\gamma1$ subunit and assembling it into multisubunit laminin oligomers. Since no alternative splicing of the $\gamma1$ mRNA in the E8 region and only one amino acid difference from the published sequence (Arg to Lys) were detected in C2C12 cells, the lack of reactivity with 5D3 antibody may reflect a different posttranslational modification, leading to a loss of the 5D3, but not the LT3, epitope, or perhaps differences in preparation of the laminins. It has been reported that mouse C2 myoblasts synthesized two immunologically distinct forms of laminin $\beta2$ chain (*s*-laminin), which differed by an unidentified posttranslational modification other than N-linked glycosylation [37]. Similar modification could be involved in the generation of a $\gamma1$ chain immunologically different from the one synthesized by EHS tumor cells.

In intact C2C12 myotubes, we were unable to detect a complex between integrin $\alpha7\beta1$ and laminin, either endogenous or that produced by EHS tumor cells, which bound well to the integrin *in vitro*. Cross-linking of cell surface proteins provided no evidence of interaction between laminin and integrin $\alpha7\beta1$ (Fig. 8). Since integrin occupancy and aggregation often have synergistic roles in the activation of postreceptor cellular events [38, 39], it could be postulated that integrin $\alpha7\beta1$ has a low affinity for soluble, monovalent laminin, and a multivalent ligand is required to induce a high-affinity conformation of the receptor. No cross-linking of laminin and integrin $\alpha7\beta1$ was observed, however, following attachment of C2C12 myotubes to laminin immobilized on plastic dishes in a multivalent network (Fig. 9). In this experiment, any endogenous proteins, which might have been bound to integrin $\alpha7\beta1$ and blocking its interactions with EHS laminin, should have been removed during detachment of cells with EDTA, prior to adhesion to a laminin-coated surface.

Results obtained in nonmyogenic cell types provide evidence for an active role of integrin $\alpha7$ in mediating cell adhesion to or migration on laminin. Human kidney and melanoma cells, which were immobile on laminin and devoid of endogenous integrin $\alpha7$, but expressed integrin $\beta1$, gained motility after transfection with integrin $\alpha7$B cDNA, while their adhesion to laminin was not changed [14]. In other experiments, integrin $\alpha7\beta1$ expressed in human breast carcinoma or fibrosarcoma cells promoted adhesion and motility on laminin, which was blocked by a function-perturbing antibody against integrin $\alpha7$ [12, 13]. Another anti-$\alpha7$ antibody completely and selectively blocked adhesion and migration of myoblasts on laminin-1 and, to a much lesser extent, on merosin [11]. Our findings point to important differences in the properties of integrin $\alpha7\beta1$ in solution, i.e., after extraction from the membrane with a detergent, in nonmyogenic cells or in myoblasts, and in intact myotubes, where interactions between laminin and integrin $\alpha7\beta1$ seem to be attenuated. Two possible explanations of this finding include steric hindrance imposed by the membrane environment or an inactive state of the integrin [40].

Recently, it has been reported that one of the two proteins, laminin-1 or merosin (laminin-2/-4, $\alpha2$-$\beta1/\beta2$-$\gamma1$), was required to induce myoblast differentiation, but only merosin promoted myotube stability by preventing apoptosis [23]. Since integrin $\alpha7\beta1$ binds laminin *in vitro* and because there is a switch in expression of different splice variants of both integrin subunits during myogenic differentiation, which is coincidental with the switch in expression of laminin-1 and merosin, it has been postulated that integrin $\alpha7\beta1$ could be a functional receptor for laminin-1 or merosin. Consistently, myofibers of merosin-deficient human patients and mice showed an abnormal expression and localization of $\alpha7$A/B$\beta1$D isoforms throughout the sarcolemma [41]. The localization of $\alpha7\beta1$ was intact, however, at the myotendinous junction [41]. Interestingly, an impairment of function of myotendinous junction was observed in knockout mice lacking the $\alpha7$ gene, whereas no defect in myogenesis was detected [42]. This raises the question of $\alpha7\beta1$ integrin being a receptor mediating the functions exerted by laminin-1 or merosin outside the myotendinous junction. Moreover, although fusion-deficient myoblast clones expressed neither laminin-1 nor merosin, either one of the two proteins added to the culture medium induced their fusion [23]. Similarly, exogenous merosin converted unstable myotubes, which were devoid of merosin, to a stable phenotype [23]. This suggested that a putative receptor for laminin/merosin, involved in myoblast fusion and further conferring myotube stability, could bind to both endogenous and exogenous ligands in intact cells. Based on the findings presented here, this receptor does not seem to be integrin $\alpha7\beta1$.

We thank Dr. Dale R. Abrahamson for generously providing 5D3 anti-laminin antibody and Dr. Stephen J. Kaufman for helpful suggestions and the anti-integrin $\alpha7$ antibody. We also thank Dr. Martha Vaughan for critical review of this manuscript.

REFERENCES

1. Hynes, R. O. (1992) *Cell* **69,** 11–25.
2. Clark, E. A., and Brugge, J. S. (1995) *Science* **268,** 233–238.
3. Yamada, K., and Miyamoto, S. (1995) *Curr. Opin. Cell Biol.* **7,** 681–689.
4. Song, W. K., Wang, W., Foster, R. F., Bielser, D. A., and Kaufman, S. J. (1992) *J. Cell Biol.* **117,** 643–657.
5. von der Mark, H., Durr, J., Sonnenberg, A., von der Mark, K., Deutzmann, R., and Goodman, S. L. (1991) *J. Biol. Chem.* **266,** 23593–23601.
6. Kil, S. H., and Bronnerfraser, M. (1996) *Int. J. Dev. Neurosci.* **14,** 181–190.
7. Bao, Z. Z., Lakonishok, M., Kaufman, S., and Horwitz, A. F. (1993) *J. Cell Sci.* **106,** 579–590.
8. Ziober, B. L., Vu, M. P., Waleh, N., Crawford, J., Lin, C.-S., and Kramer, R. H. (1993) *J. Biol. Chem.* **268,** 26773–26783.
9. Collo, G., Starr, L., and Quaranta, V. (1993) *J. Biol. Chem.* **268,** 19019–19024.
10. Song, W. K., Wang, W., Sato, H., Bielser, D. A., and Kaufman, S. J. (1993) *J. Cell Sci.* **106,** 1139–1152.
11. Crawley, S., Farrell, E. M., Wang, W., Gu, M., Huang, H.-Y., Huynh, V., Hodges, B. L., Cooper, D. N. W., and Kaufman, S. J. (1997) *Exp. Cell Res.* **235,** 274–286.
12. Yao, C.-C., Ziober, B. L., Squillace, R. M., and Kramer, R. H. (1996) *J. Biol. Chem.* **41,** 25598–25603.
13. Ziober, B. L., Chen, Y-Q., and Kramer, R. H. (1997) *Mol. Biol. Cell* **8,** 1723–1734.
14. Echtermeyer, F., Schöber, S., Pöschl, E., von der Mark, H., and von der Mark, K. (1996) *J. Biol. Chem.* **271,** 2071–2075.
15. Timpl, R., and Brown, J. C. (1994) *Matrix Biol.* **14,** 275–281.
16. Wewer, U. M., and Engvall, E. (1994) *Methods Enzymol.* **245,** 85–104.
17. Kühl, U., Timpl, R., and von der Mark, K. (1982) *Dev. Biol.* **93,** 344–354.
18. Foster, R. F., Thompson, J. M., and Kaufman, S. J. (1987) *Dev. Biol.* **122,** 11–20.
19. Goodman, S. L., Risse, G., and von der Mark, K. (1989) *J. Cell. Biol.* **109,** 799–809.
20. von der Mark, H., and Öcalan, M. (1989) *Differentiation* **40,** 150–157.
21. Sanes, J. R., Schachner, M., and Couvalt, J. (1986) *J. Cell. Biol.* **102,** 420–431.
22. Schuler, F., and Sorokin, L. M. (1995) *J. Cell Sci.* **108,** 3795–3805.
23. Vachon, P. H., Loechel, F., Xu, H., Wewer, U. M., and Engvall, E. (1996) *J. Cell Biol.* **134,** 1483–1497.
24. Mercurio, A. M. (1995) *Trends Cell Biol.* **5,** 419–423.
25. Sanes, J. R., Engvall, E., Butkowski, R., and Hunter, D. D. (1990) *J. Cell Biol.* **111,** 1685–1699.
26. Martin, P. T., Ettinger, A. J., and Sanes, J. R. (1995) *Science* **269,** 413–416.
27. Zolkiewska, A., Nightingale, M. S., and Moss, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11352–11356.
28. Okazaki, I. J., Zolkiewska, A., Nightingale, M. S., and Moss, J. (1994) *Biochemistry* **33,** 12828–12836.
29. Zolkiewska, A., and Moss, J. (1993) *J. Biol. Chem.* **268,** 25273–25276.
30. Zolkiewska, A., and Moss, J. (1995) *J. Biol. Chem.* **270,** 9227–9233.
31. Cooper, D. N. W., and Barondes, S. H. (1990) *J. Cell Biol.* **110,** 1681–1691.
32. Cooper, D. N. W., Massa, S. M., and Barondes, S. H. (1991) *J. Cell Biol.* **115,** 1437–1448.
33. Abrahamson, D. R., Irwin, M. H., St. John, P. L., Perry, E. W., Accavitti, M. A., Heck, L. W., and Couchman, J. R. (1989) *J. Cell Biol.* **109,** 3477–3491.
34. Engvall, E., and Wewer, U. M. (1996) *J. Cell. Biochem.* **61,** 493–501.
35. Abrahamson, D. R., and St. John, P. L. (1993) *Kidney Int.* **43,** 73–78.
36. Ljubomov, A. V., Burgeson, R. E., Butkowski, R. J., Michael, A. F., Sun, T.-T., and Kenney, M. C. (1995) *Lab. Invest.* **72,** 461–473.
37. Green, T. L., Hunter, D. D., Chan, W., Merlie, J. P., and Sanes, J. R. (1992) *J. Biol. Chem.* **267,** 2014–2022.
38. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) *J. Cell Biol.* **131,** 791–805.
39. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) *Science* **267,** 883–885.
40. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Biol.* **11,** 49–99.
41. Vachon, P. H., Xu, H., Liu, L., Loechel, F., Hayashi, Y., Arahata, K., Reed, J. C., Wewer, U. M., and Engvall, E. (1997) *J. Clin. Invest.* **100,** 1870–1881.
42. Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Poschl, E., and von der Mark, K. (1997) *Nat. Genet.* **17,** 318–324.

Received September 12, 1997
Revised version received January 5, 1998