Alternative Splice Variants of $\alpha_7\beta_1$ Integrin Selectively Recognize Different Laminin Isoforms*

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The integrin $\alpha_7\beta_1$ occurs in several cytoplasmic ($\alpha_7\alpha_2$, $\alpha_7\alpha_6$) and extracellular splice variants ($\alpha_7\alpha_1$, $\alpha_7\alpha_3$), which are differentially expressed during development of skeletal and heart muscle. The extracellular variants result from the alternative splicing of exons X1 and X2, corresponding to a segment within the putative ligand binding domain. To study the specificity and affinity of the X1/X2 variants to different laminin isoforms, soluble $\alpha_7\beta_1$ complexes were prepared by recombinant coexpression of the extracellular domains of the $\alpha_7$- and $\beta_1$-subunits. The binding of these complexes to purified ligands was measured by solid phase binding assays. Surprisingly, the alternative splice variants revealed different and specific affinities to different laminin isoforms. While the $\alpha_7\alpha_1$ variant bound much more strongly to laminin-1 than the $\alpha_7\alpha_3$ variant, the latter showed a high affinity binding to laminins-8 and -10/11. Laminin-2, the major laminin isoform in skeletal muscle, was recognized by both variants, whereas none of the two variants were able to interact with laminin-5. A specific blocking antibody inhibited the binding of both variants to all laminins tested, indicating the involvement of common epitopes in $\alpha_7\alpha_1\beta_1$ and $\alpha_7\alpha_3\beta_1$. Because laminin-8 and -10/11 as well as $\alpha_7\alpha_1$ are expressed in developing skeletal and cardiac muscle, these findings suggest that $\alpha_7\alpha_1\beta_1$ may represent a physiological receptor with novel specificities for laminin-8 and -10.

The laminin-binding integrin $\alpha_7\beta_1$ is the major laminin receptor of skeletal, cardiac, and smooth muscle cells (for review, see Ref. 1), but it has also been detected in some human melanoma cells (2) and glioblastoma cells (3). In skeletal muscle, it is located predominantly in myotendinous and in neuromuscular junctions (4, 5) and in the sarcolemma (6). Experimental evidence from several laboratories indicates that $\alpha_7\beta_1$ integrin is involved in laminin-induced migration of skeletal muscle myoblasts (7, 8) and other cells: cell motility of melanoma, HEK293 cells (9, 10), and MCF7 cells (11) on laminin-1 and -2 was considerably enhanced after transfection with $\alpha_7\beta_1$ integrin expression vectors. The critical role of $\alpha_7\beta_1$ in muscle function became evident after inactivation of both alleles of the $\alpha_7$ gene itga7 in mice. Deficient mice developed a novel form of myopathy, accompanied by disruption of the myotendinous junctions (12). Subsequently, human patients were identified with similar defects associated with mutations in the Itga7 gene (13).

The $\alpha_7\beta_1$ integrin subunits are expressed in two cytoplasmic and two extracellular splice variants in human and mouse tissues (14–16), the expression of which is developmentally regulated. The cytoplasmic variant $\alpha_7\beta_1$ is expressed in cardiac and smooth muscle, skeletal myoblasts, and embryonic and adult skeletal muscle, whereas the $\alpha_7\alpha_1$ variant is expressed only in mature skeletal muscles but not in cardiac muscle (6). The extracellular variants $\alpha_7\alpha_1$ and $\alpha_7\alpha_3$ result from alternative splicing of exons coding for a region between the homology repeats III and IV, located in the putative $\beta$-propeller domain of $\alpha_7$, and represent part of the putative ligand binding site. The X1 and X2 splice variants are expressed in equal amounts in mouse skeletal myoblasts and adult heart, whereas in adult skeletal muscle, mainly the $\alpha_7\alpha_3$ splice variant can be found (14, 15). The specific roles of these splice variants are still unclear. After experimental clustering of acetylcholine receptors on the surface of $\alpha_7\alpha_1$- or $\alpha_7\alpha_3$-transfected C2C12 myotubes by laminin-1, only the $\alpha_7\alpha_3$ was detected within the acetylcholine receptor clusters (18). Both splice variants bind to laminin-1- and laminin-2-coated surfaces when expressed in HEK293 cells; however, $\alpha_7\alpha_1$B promoted cell migration only on laminin-2, and $\alpha_7\alpha_3$ stimulated motility on both substrates (10). Further, the cell-specific environment seems to have a critical impact on binding (10, 17).

Although $\alpha_7\beta_1$ integrins bind to laminin-1 and its E8 fragment in vitro (3, 19), this isoform does not occur in skeletal muscle in vivo (20–22). Laminin-2 appears to be the major $\alpha_7\beta_1$ ligand in skeletal muscle in vivo; however, nothing is known about $\alpha_7\beta_1$ interactions with other laminin isoforms that also occur in myogenic tissues, such as laminin-8 (23) and laminin-10 (21, 24–26). Laminin-8, composed of $\alpha_4$, $\beta_1$, and $\gamma_1$ chains, and laminin-10 ($\alpha_5$, $\beta_1$, $\gamma_1$), which were originally identified in endothelial cell basement membranes (21, 23, 24, 27), are also expressed in muscle fiber basement membranes during embryogenesis. Both isoforms are down-regulated during development, remaining at the neuromuscular junction in the mature tissues where they combine with laminin $\beta_2$ and $\gamma_1$ chains to form laminin-9 and -11, respectively (21, 28). The 200–240-kDa $\alpha_4$ chain that forms laminin-8 together with $\beta_1$ and $\gamma_1$ subunits (29) was localized in fetal and newborn neuromuscular junctions and the endoneurium of intramuscular nerves but disappears in mature muscle, whereas it remains in the capillary endothelial junctions (21). Furthermore, laminin $\alpha_4$ and $\alpha_5$ chains reappear to compensate for the lack of laminin-2 in basement membranes of newly formed myotubes in focal le-
specificities in the mouse and human integrin family (21). Recent data show that laminin-8 and -10 are up-regulated in the basement membranes of newly formed myotubes during regeneration of normal adult muscle (28).

In order to define the affinity and specificity of α7β1 integrin and its extracellular splice variants for different laminins, soluble α7X1β1 and α7X2β1 complexes were expressed recombinantly in HEK293 cells, and affinities for laminin isoforms were analyzed using a solid phase binding assay. The extracellular domains of integrin β1 and α7 formed heterodimers and were secreted to the culture medium. Surprisingly, X1 and X2 variants showed distinct binding affinities for different laminin isoforms. The X2 splice variant showed a high affinity binding to laminin-1 and its E8 fragment but did not bind laminins-8 and -10. The X1 variant, in contrast, specifically recognized laminin-8 and -10, and to a lesser extent, it also recognized laminin-1. Both X1 and X2 showed similar affinities for laminin-2, whereas laminin-5 was not recognized by either splice variant. Because striking similarities exist between the expression patterns of the α7X1β1 splice variant and laminins-8 and -10, we assume that α7X1β1 represents a potential receptor for laminin-8 and -10 during early steps in embryogenesis.

**Experimental Procedures**

**Purification of Laminins and Other Matrix Proteins—**Laminin-1 was purified from the murine Engelbreth-Holm-Swarm tumor (30), and laminin-2 was extracted from mouse heart muscle and purified as described previously (31). Laminin-5 was prepared from human SSC25 cells (32) (kindly provided by Dr. J. Eble, University of Münster, Germany). Bovine laminin-8 was isolated from conditioned medium of bovine aortic endothelial cells endothelial cells as described in Ref. 23. Laminin-10/11 was purified from human placenta by affinity chromatography using the anti-α7 antibody, 4C7 (33). Collegen I and fibronectin were purchased from Sigma.

**Generation of Expression Vectors for Soluble Integrin Chains—**The cloning of the murine α7N2X variant was described earlier (9) with the full-length cDNA corresponding to positions 14–8389 of GenBank accession number GB:NM_008398. An additional RGS-His tag (Qiagen) was introduced at the C-terminal end of the extracellular domain (position 3280) using a PCR-based strategy, resulting in the construct pCMVsis/1-E-Strep/H9251. The extracellular domain of the X1 variant (position 57–190) of the α7 integrin after deletion of the N-terminal extracellular domain (position 1–56) was generated by a reverse transcription-PCR fusion strategy using overlapping primers corresponding to the X1-specific sequence (14, 15) in combination with N- and C-terminal primers. The resulting 1660-bp N-terminal fragment was cloned and fused with the C-terminal half containing the RGS-His tag in pUC18/α7C-His. The plasmid pBSmβ1-R contains the full-length cDNA of the murine β1, cDNA (a kind gift of S. Fassler and R. Faessler), and sequences coding for the C terminus of the ectodomain were amplified by PCR using Vent Polymerase (Biolabs) with an additional Strep-tag sequence, SAWHR-PQFGG (34), introduced at the C terminus at position 2278. The 0.44-kb fragment, corresponding to positions 1880–2278 (GenBank accession number GB:mmintb3), was cloned and fused via an internal Bgl II site with the N-terminal Hpa I/Bgl II fragment (position 57–190) of pBSmβ1-R, resulting in pBSβ1-E-Strep. All clones were verified by restriction mapping and sequencing. The inserts from pUC18/α7C-His and pUC18/α7C-His, coding for His-tagged α7, ectodomains, were isolated by XbaI/Hpal digestion and recloned in the corresponding sites of the vector pCMVα (35), resulting in pCMVα/α7C-His and pCMVα/α7C-His. The insert from pBSβ1-E-Strep was recloned in the expression vector pCNA5 (Invitrogen) by the use of RNase X1. The purified soluble α7β1 integrin was then obtained using nickel-nitrilotriacetic acid affinity chromatography (Fig. 1). The chain composition of the purified complexes was tested by immunoblotting using specific antibodies to α7 and β1 subunits (Fig. 1, B and C). The estimated sizes for the soluble α7β1 (97 kDa) and α7β2 (95 kDa) subunits corresponded with the calculated sizes of the expressed domains, whereas the diffuse band obtained with the β1 subunit (110–120 kDa) is indicative for a high level of glycosylation. Although most clones produced and secreted β1 chains in excess, both truncated subunit chains appeared in comparable amounts after purification, although the presence of small amounts of single chains cannot be excluded (Fig. 1A). Mn2+ was added to purified complexes and was always present during experiments. The absence of Mn2+ resulted in a loss of stability of the α7β1 complexes; thus Mn2+ could not be replaced by other divalent anions. Up to 1.5 mg of soluble complexes was isolated from 1 liter of conditioned medium.

The abbreviations used are: TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; LN, laminin.
α7X2β1 Binds to Laminin-1 with Higher Affinity Than α7X1β1—The binding of the α7X1β1 and α7X2β1 variants to laminin-1 was investigated by a solid phase binding assay on microtiter dishes coated with purified laminin-1 at varying concentrations (2.5–20 μg/ml). Bound receptor was detected by antibodies specific for the α7 (not shown) or the β1 chain (Fig. 2A). The binding was saturable and revealed a higher affinity of α7X2β1 for laminin-1 as compared with α7X1β1. The difference was further substantiated by calculations of the Kd values using Scatchard plot and saturation plot analysis (37). Amounts of bound ectodomain complexes were calculated from calibration curves established by a sandwich ELISA (see “Experimental Procedures”). The affinity of α7X1β1 for laminin-1 (Kd ~ 17 × 10⁻⁹ m) is significantly lower than the affinity of the α7X2β1 variant for the same ligand (Kd ~ 3 × 10⁻⁹ m). Corresponding differences were also seen after the binding of variable concentrations of soluble receptors of α7X1β1 and α7X2β1 to constant concentrations (10 μg/ml) of laminin-1 (Fig. 2B). Similarly, α7X2β1 showed a significantly higher affinity for the E8 fragment of laminin-1 than the X1 splice variant (Fig. 3). In contrast, both complexes bound to laminin-2 to a comparable extent at high concentrations (100 μg/ml) of receptors (Figs. 2A and 3), which is in agreement with a previous study where we found comparable levels of binding of α7X1 and α7X2-expressing HEK293 cells to laminin-2 (10). Low concentrations of receptor revealed a higher affinity of α7X1β1 for LN-2 (Fig. 2B). Neither α7X2β1 nor α7X2β1 bound to laminin-5 (Fig. 3), which is recognized by α7β1 and α7β4 (32, 38, 39). No binding of either α7 splice variant to collagen I, fibronectin, or bovine serum albumin could be detected. The presence of EDTA completely inhibited the binding of receptor variants to all laminins (data not shown).

α7X2β2, but Not α7X2β1, Specifically Recognizes Laminin-8 and Laminin-10/11—Surprisingly, binding characteristics of the α7X1 and α7X2 splice variants to laminins-8 and -10/11 were opposite to those on laminin-1. Only the α7X1β1 complex, but not the α7X2 variant, bound to these laminins (Fig. 3). The affinity of α7X1β1 for laminin-8 (Kd ~ 2 × 10⁻⁹ m) is comparable with the affinity of α7X2β1 for laminin-1. At similar coating concentrations of laminin isoforms, the binding of α7X1β1 to laminins-8 and -10/11 was considerably higher than to laminin-1 (Fig. 3). This was confirmed in a binding assay on microtiter plates coated with serial dilutions of laminins (Fig. 2A) assuming comparable coating efficiency as shown recently (33).

The α7-specific antibody 6A11 was previously shown to inhibit α7β1-mediated adhesion of transfected HEK293 cells (10). The addition of this blocking antibody inhibited the binding of α7X2β1 to laminin-1 as well as α7X1 to laminins-1, -8, and -10/11 (Fig. 4), indicating that common interaction sites are involved in ligand binding of both splice variants.

DISCUSSION

In this study, we present for the first time evidences for differential specificity of the extracellular splice variants of α7 integrin for laminin isoform ligands. Laminins as biologically active components of basement membranes have been shown to regulate cell adhesion and spreading, cell proliferation, differentiation, and migration. Many in vitro studies on the biologi-
cal roles of laminin have been performed using laminin-1, predominantly found in embryonic and fetal tissues but absent from many adult tissues including skeletal muscle (20-22). At present, 15 distinct laminins have been described in different basement membranes (26, 29, 40, 41). The major laminin isof- form of skeletal muscle is laminin-2, whereas laminin-4 is restricted to neuromuscular junctions (25). Basement membranes surrounding embryonic myofibers also contain laminins-8 and -10, which are down-regulated at this site with development. A number of β1 integrins, including α7β1, α6β1, α3β1, α9β1, α6β1, and α6β1 have been shown to be involved in cellular interactions with laminins, but only α6β1 and β1β1 bind exclusively to laminins: α3β1 recognizes laminin-1 and -2 (10, 11), α6β1 binds to laminin-1, -2, and -10 (10, 42, 43), whereas α9β1 recognizes laminins-5 and -10 (32, 42). The integrin α-subunits α9, α6, and α7 are structurally highly homologous, lack I-domains at the N terminus, and are expressed as two extracellular splice vari- ants (X1, X2). Alternative cytoplasmic domains (A, B) are also generated by differential splicing of integrin α9 and α7 genes. However, little was known about the affinity of α7X1 and α7X2 splice variants for laminin isoforms. The α7X1β1 and α7X2β1 integrin complexes used in this study were prepared as recombinant, soluble ectodomains lacking the cytoplasmic and transmembrane domains. Despite the absence of these domains, the extracellular domains of α7X1 or α7X2 subunits formed stable heterodimeric complexes with the truncated β1 subunit, which apparently retained their native structure and ligand binding capacity. Heterodimerization of α7 and β1 subunits was proven by immunoprecipitation of the complexes with antibodies to α7, as well as by affinity chromatography of the tagged α7X1 or α7X2 subunits. Obviously, the extracellular domains of α7X1/α7X2 and β1 contain sufficient structural information for their assembly into stable heterodimers. Alternatively, soluble α7β1 heterodimers devoid of cytoplasmic and transmembrane domains have been prepared by coupling jun and fos leucine zippers to the C termini of subunits to stabilize the complex (32). In that case, the use of dimerization domains stabilized the complex and also allowed binding studies in the presence of different cations. The essen- tial addition of Mn2+ and Mg2+ for the stabilization of the soluble α7β1 complexes points into this direction. The method used in this study may be useful for the detailed analysis of receptor-ligand interactions by mutagenesis studies and epitope mapping. The binding of α7X1β1 to laminin-8 and -10/11, as well as the interaction of both variants to laminin-1 and -2, could be completely inhibited by the α7 blocking antibody, 6A11, which was shown previously to block α7-integrin-mediated binding of cells to laminin-1/E8 fragment (10). This indicates that the ligand binding of α7X1/α7X2 variants with laminin isoforms share common epitopes in the extracellular domain, whereas specificity is defined by the X1/X2 region located between homology repeats III and IV in the β-propeller of the α7 chain (44). Nevertheless, it cannot be excluded that the blocking effects of the antibody may result from steric hindrance or by allosteric effects. The soluble α7X1β1 showed a higher affinity for laminin-1 and laminin-1/E8 fragment than the α7X2β1 complex. In accord- ance with this finding, α7X1-transfected HEK293 cells are characterized by an increased motility on laminin-1 as com- pared with α7X2-transfected cells, although adhesion rates were about equal for both splice variants (10). After prolonged attachment times, however, both α7X1- and α7X2-transfected cells may have reached saturation in adhesion to laminin-1. Furthermore, there is ample evidence that the cellular environ- ment of β1 integrins greatly affects their affinity and specific- ity of binding. Thus, the interpretation of the role of distinct integrins in cell adhesion may be obscured by the presence of other integrins on the cell surface. For example, Zibor et al. (17) demonstrated that α7X1-transfected MCF7 cells bound laminin-1 only after activation with the β1-activating antibody, TS/16, whereas α7X2-transfected HT1080 cells constitutively bound laminin-1. These data indicated a conformational de- pendence of the laminin binding site in the α7X1 extracellular domain, which can be regulated by β1 antibodies and by divalent cations, in particular Ca2+, Mg2+, or Mn2+. Recently, antibody studies indicated laminin-induced conformational changes of α7 integrins in muscle cells (45). α7β1 integrins bind only to native laminins and require the C-terminal portions of laminin α-, β-, and γ-chains in their native trimeric conformation for binding (19, 46, 47). Since the laminin β1 and γ1 chains are identical in laminins-1, -2, -8, and -10, differences in the laminin α-chains must account for the different affinities of the α7X1 and α7X2 variants for these lami- nins. There is only moderate sequence homology between the C-terminal portions of laminin α6 and α3 chains that comprise the globules of laminin-8 or -10/11, respectively. The degree of homology between laminin α4 and α6 is not higher than that between laminin α4 and α3 or α1 chains; it is therefore surpris- ing that the α7X2 integrin recognizes laminin-1 and -2 but not laminin-8 and -10/11, while α7X1 recognizes all four laminins, although at different affinities. Neither α7X1 nor α7X2 bind to laminin-5, however, which contains the laminin α3 chain. Further studies on the integrin-binding epitopes of the laminin isoforms will be necessary for the characterization of the structural basis of specificity for interactions with integrins. The question remains whether the absence of the cytoplas- mic domains from the soluble complexes may affect the affinity and specificity of the extracellular domains to their ligands. There is no precedence, however, for a direct impact of the cytoplasmic domains of α-subunits on the ligand binding speci- ficity or affinity of β1 integrins. The cytoplasmic domains of α-subunits are involved in the transduction of extracellular signals to the cytoskeleton and intracellular signaling pathways, which regulate cellular responses and pathways regulating cellular responses (48-51). Deletion of the entire cytoplasmic domain of α7 integrin has been shown to have no effect on the adhesion of transfected HEK293 cells to laminins-1 or -2, but it does alter cell migration and phospho- rylation reactions (52). It remains to be elucidated whether the laminin isoform-specific interactions of the soluble α7X1β1 and α7X2β1 variants reflect their affinities in vivo or whether they are modulated in vivo depending on the cell type in which they are expressed, similar to the cases of α6β1 (17) or α9β1 (53). Cell type-dependent modulation of binding affinities, however, is seen in tumor cells or after ectopic expression of integrins in cell lines. So far, cells expressing only the α7X1 splice variant have not been identified, but attempts are in progress to iden- tify and to isolate such cells from muscle tissue to verify the affinity of the α7 splice variant for laminin-8 and -10. The specific affinity of the α7X1β1 variant for laminins-8 and -10/11 suggests that α7X1 may play a potential role in developing mouse skeletal muscle and also during situations associated with the reexpression of these laminins in regenerating muscle (21). α7β1 integrin is also strongly expressed in smooth muscle that lines the walls of arteries. In view of the wide- spread distribution of laminin-8 and -10/11 in embryonic muscle including the sarcolemma, neuromuscular junctions, and the endoneurium, it seems more likely that the α7X1 integrin splice variant is involved in heterologous cellular interactions in these locations. Therefore, the data presented may indicate that α7X1β1 represents a potential receptor for laminins-8 and -10 during early embryogenesis, whereas α7X2β1 in later stages
is mainly involved in the functions of skeletal and heart muscle.

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