Fibulin-2 Binds to the Short Arms of Laminin-5 and Laminin-1 via Conserved Amino Acid Sequences*

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Epithelial cell-specific laminin-5, consisting of three chains, α3, β3, and γ2, is a component of the anchoring filament that traverses the lamina lucida beneath the hemidesmosomes of epidermal cells and functions to link these cells to the basement membrane. We have studied the molecular interaction between laminin-5 and extracellular matrix proteins using recombinant proteins and synthetic peptides. Affinity chromatography assays with recombinant fragments of the laminin γ2 short arm identified a 195-kDa binding protein in the conditioned media from the mouse epidermal cell line Pam 212 and from primary dermal fibroblasts. This molecule was identified by Western blotting as fibulin-2, a recently identified extracellular matrix protein. Using deletion mutants and various synthetic peptides in competition assays, the 9-amino acid sequence SADFSVHKI (residues 199–207) in domain IV of the γ2 chain was defined as a critical site for fibulin-2 binding. An anti-γ2 antibody co-immunoprecipitated fibulin-2 from the conditioned media, further confirming the interaction of fibulin-2 with laminin-5. Fibulin-2 was also found to interact with laminin-1 (α1β1γ1) through a region (residues 654–665) of the α1 chain short arm whose sequence is similar to that of the fibulin-2 binding site of the γ2 chain. Together these results suggest that fibulin-2 functions to bridge laminin-1 and laminin-5 with other extracellular matrix proteins, providing a linkage between the cell surface and the basement membrane.

At the dermal-epidermal junction, there is stable attachment of epithelia to the underlying stroma through various protein-protein interactions. Electron microscopy and immunohistochemical studies have defined the topographical linkage between hemidesmosomes on the basal surface of epithelium, anchoring filaments, and anchoring fibrils. These structures form an extended network, which surrounds the stromal fibers and inserts into the basement membrane. Hemidesmosomes of basal keratinocytes contain several molecules including BP180, BP230, HD1, and integrin α6β4. The anchoring filaments contain laminin-5 (kalinin/nicain, epligrin) and colocalize with hemidesmosomes at the suprabasal basement membrane. The basement membrane components laminin-1, type IV collagen, and nidogen/entactin and the anchoring fibrils consisting of type VII collagen are located on the dermal side of the basement membrane (1–3).

Mutations in the genes for the components of the dermal-epidermal junction in human patients with skin blister-forming disease have revealed the importance of these protein linkages in maintaining the structural stability of the dermal-epidermal junction. Mutations in BP180 (4), integrin β4 (5), laminin-5 (6–9), and type VII collagen (10–12) have been identified. Acquired skin blister-forming diseases have also been shown to be due to autoantibodies to BP180 (13), laminin-5 (14), and type VII collagen (15).

Basement membrane components have been shown to interact with each other and self-assemble to form a supramolecular network. Laminin-1 polymerizes through interactions at the N-terminal short arms of the monomeric molecules to form a hexagonal array of molecules (16). Nidogen is also a crucial molecule required for network formation since it binds several components of basement membrane including laminin-1, type IV collagen, and perlecian (17–19). Fibulin-1 (BM-90) and fibulin-2 were recently identified as a family of extracellular matrix proteins that interact with the laminin-1-nidogen complex, type IV collagen, and fibronecin (20–23). The interaction of fibulins with multiple components of the extracellular matrix suggests that they function as mediators of supramolecular assembly at the basement membrane.

Epithelial cell-specific laminin-5 consists of three chains, α3, β3, and γ2, and is an adhesive substrate for keratinocytes in vitro (24). Recently, both α6β4 and α6β1 integrins were identified as cellular receptors for laminin-5 (25–29). Laminin-5 forms a disulfide-bonded complex with laminin-6 (α3β1γ1) (30), possibly via the N-terminal globular domain VI of the laminin β3 chain. The N-terminal globular domain VI of the laminin β3 chain may be involved in the complex formation, since there is an uncoupled cysteine residue within this domain (31, 32).

In order to study the interaction between laminin-5 and other extracellular matrix components, proteins in the culture medium of Pam 212 epidermal cells were screened for binding recombinant laminin chains by affinity chromatography. Fibulin-2, which is prominently expressed in skin and heart, was found to bind laminin-5 through the short arm of the γ2 chain and we have identified a 9-amino acid sequence in domain IV of the γ2 chain critical for this binding. We have also found that fibulin-2 binds to laminin-1 via the N terminus of the α1 chain, a site showing sequence homology to the 9-amino acid sequence of the γ2 chain. Together these results suggest that fibulin-2 functions in assembling the laminin network in the basal lamina at the dermal-epidermal junction in bridging laminin-1 and laminin5 with other matrix proteins.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Reagents—The murine epidermal cell line Pam 212 (33) and the human epidermoid carcinoma cell line A431 were obtained from ATCC (Bethesda, MD). Primary cultures of mouse dermal fibroblasts were isolated from newborn PVD/N mouse. All cells were maintained with 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium (Life Technologies Inc.). Anti-mouse fibulin-2 antiserum was made by immunizing rabbits with recombinant fibulin-2 (34) kindly provided by Dr. R. Timpl (Max-Planck-Institut für Biochemie, Munich, Germany). Anti-laminin γ2 chain antiserum was prepared as

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described previously (35). Anti-mouse type IV collagen antibody was made with Engelbreth-Holm-Swarm-derived type IV collagen in rabbits and purified using type IV collagen-coupled Sepharose column. Anti-human thrombospoindin antibody was a generous gift from Dr. D. D. Roberts (NCI, NIH, Bethesda, MD) (36). Laminin-1 was prepared from the Engelbreth-Holm-Swarm tumor and purified as described previously (37). Bacterial collagenase form III was purchased from Advanced Biotechnologies Inc.

Recombinant Proteins and Synthetic Peptides—cDNAs for murine laminin α2, γ2, and β3 chains were produced as reported previously (32, 35, 38). cDNA fragments generated by restriction enzymes or polymerase chain reaction were subcloned into either the pGEX-2T or -4T bacterial expression vector (Pharmacia Biotech Inc.). Recombinant proteins were expressed and purified as described previously (39). These recombinant proteins include: γ2-r1 (residues 17–306), γ2-r2 (residues 129–306), γ2-r3 (residues 197–306), γ2-r4 (248–306), γ2-r5 (residues 197–247) of the γ2 chain, β3-r1 (residues 18–253) of the β3 chain, α2-r1 (residues 23–242) of the α2 chain. All constructs used in these studies were confirmed by automated DNA sequencing (model 373A, Applied Biosystems, Foster City, CA).

Synthetic peptides, γ2-pC and γ2-pN, were synthesized with a peptide synthesizer (Applied Biosystems, model 431A) by the t-butyloxycarbonyl-based solid-phase strategy (40). All other synthetic peptides were manually synthesized by the Fmoc (9-fluorenlymethoxycarbonyl)-based solid-phase strategy and prepared as the C-terminal amide form as described previously (41). All synthetic peptides were purified by reverse phase high performance liquid chromatography. The purity and identity of the synthetic peptides were confirmed by analytical reverse phase high performance liquid chromatography and amino acid analysis. The location and amino acid sequences of the synthetic peptides from the γ2 chain are listed in Table I and Fig. 3. Synthetic peptides listed in Fig. 7B include: pN-21, 9 amino acids (residues 190–207) of the γ2 chain; pO1–654, 12 amino acids (residues 654–665) of the α1 chain; pO1–279, 9 amino acids (residues 279–287) of the α1 chain; pO1–461, 9 amino acids (residues 461–469) of the β1 chain with two arginine residues added to increase the solubility of the peptide; pY1–587, 9 amino acids (residues 587–595) of the γ1 chain with two added arginine residues to increase the solubility of the peptide; and pO2–287, 9 amino acids (residues 287–295) of the α2 chain.

Affinity Chromatography and Competition Assays—The recombinant proteins fused to glutathione S-transferase (GST) were bound to glutathione-agarose (Pharmacia) at 0.2 mg/ml. Laminin-1 prepared from Engelbreth-Holm-Swarm tumor was coupled to CNBr-activated Sepharse beads (1 mg/ml) (Pharmacia). Pam 212 cells and murine dermal fibroblasts were labeled with 50 μCi of [35S]methionine (ICN, Costa Mesa, CA) in methionine-free Dulbecco’s modified Eagle’s medium supplemented with 5 mM 2-butoxyethanol for 4 h. The conditioned media were adjusted to 2 mM phenylmethylsulfonyl fluoride and centrifuged at 3,000 revolutions/min for 10 min. These supernatants were stored at −20 °C until use. The conditioned media (500 μl) were incubated with 30 μl of affinity beads for 2 h with rotary shaking at 4 °C. Following three washings with 1 ml of 0.1% Triton X-100, phosphate-buffered saline, 2 mM phenylmethylsulfonyl fluoride, the proteins bound to the affinity beads were extracted with SDS-sample buffer. The samples were boiled with or without 100 mM dithiothreitol, analyzed on 4–12% SDS-PAGE, and treated with EDTA for 20 min at room temperature. The eluted proteins were precipitated with 10% trichloroacetic acid for 30 min on ice with 10 mg of bovine serum albumin added as a carrier. After centrifugation at 30,000 × g for 15 min at 4 °C, the pellets were washed with 1 ml of 0.5% trichloroacetic acid once and then washed two times with 1 ml of acetone. These pellets were resuspended in sample buffer and analyzed by SDS-PAGE. The identity of the 195-kDa protein as fibulin-2.

Sequence Analysis—Protein sequence analysis were performed using a software package from the University of Wisconsin Genetics Computer Group; optimal alignment was provided by the program BESTFIT.

RESULTS

Screening of Extracellular Proteins Bound to the Short Arms of Laminin-5—Since the short arms of laminins have been shown to interact with other basement membrane proteins, we examined whether additional extracellular matrix proteins bind to the short arms of the γ2 and β3 chains of laminin-5 using recombinant laminin chains. [35S]Methionine-labeled conditioned media from murine epidermal Pam 212 cells were incubated with various recombinant laminin proteins-coupled to agarose beads. After washing, bound proteins were eluted and analyzed on SDS-PAGE. The N terminus of the γ2 chain short arm bound a protein with an apparent molecular mass of 195 kDa, whereas there was no protein binding to either the short arm of the β3 chain of laminin-5 or the α2 chain of laminin-2 (Fig. 1A, lanes 1–3). Binding of a 195-kDa molecule to the γ2 chain short arm was also observed with the conditioned media from mouse dermal fibroblasts (Fig. 1A, lane 5). Since 5 mM EDTA abolished the binding, this interaction was likely dependent on a divalent cation (Fig. 1A, lane 4). Electrophoresis containing 6 μl urea showed a single protein band, strongly suggesting that this 195-kDa band consists of a single molecule (Fig. 1B). Furthermore, the shift of the molecular size from 195 kDa to ~600 kDa under non-reducing conditions suggested that the 195-kDa molecule might form a disulfide-bonded homotrimer (Fig. 1B). Digestion of this 195-kDa protein with collagenase did not cleave this protein, suggesting it does not contain a collagenous domain (Fig. 1C, lanes 3 and 4).

Fibulin-2 Binding to the Recombinant γ2 Chain Short Arm—Judging from its molecular size and its ability to form a homotrimer, we speculated that this non-collagenous 195-kDa extracellular protein might be either fibulin-2 (21) or thrombospondin (36). To test these possibilities, we used antibodies to both fibulin-2 and thrombospondin in Western blotting. The 195-kDa protein bound to the recombinant γ2 chain short arm (γ2-r1) was recognized by an antibody against mouse fibulin-2 (Fig. 2A, lane 2), but not by an antibody to thrombospondin (Fig. 2B). Furthermore, this protein that reacted with the anti-fibulin-2 antibody formed a trimer under non-reduced conditions (Fig. 2A, lane 3) and exhibited divalent cation-dependent binding to γ2-r1 (Fig. 2A, lanes 4 and 5). These data confirm the identity of the 195-kDa protein as fibulin-2.

Deletion Analysis of the Fibulin-2 Binding Site of the γ2 Chain—In order to identify the binding site for fibulin-2, a series of deletion mutants of γ2-r1 were prepared (Fig. 3A). The binding activities were monitored by affinity chromatography using conditioned media from murine epidermal Pam 212 cells (Fig. 3B). Recombinant γ2-r3 containing a deletion of domain V still interacted with fibulin-2 (Fig. 3B, lane 4), but not by an antibody to thrombospondin (Fig. 3B, lane 3). The N-terminal 51-amino acid region in domain IV (γ2-r5, residues 197–247) was active for binding but the C-terminal 59-amino acid region (γ2-r4, residues 248–306) was inactive (Fig. 3C, lanes 4 and 5). These results indicate that the active region for fibulin-2 bind-
A bead with bacterial collagenase in the presence (lanes 1) or absence (lanes 4) of 5 mM EDTA (lane 5). B, proteins bound to γ2-r1 were analyzed on 4% SDS-PAGE containing 6 x urea under non-reducing (lane 1) or reducing (lane 2) conditions. Upper arrow, electrophoretic mobility of laminin α1 chain; lower arrow, that of laminin β1 and γ1 chains. C, proteins bound to γ2-r1 (lanes 3 and 4) and immunoprecipitated type IV collagen (lanes 1 and 2) were digested on beads with bacterial collagenase in the presence (lanes 1 and 3) or in the absence (lanes 2 and 4) of 5 mM N-ethylmaleimide. The pre-stained molecular size markers 199, 120, 87, and 47 kDa were used.

The synthetic peptide γ2-pN (residues 197–226) showed inhibition of fibulin-2 binding to γ2-r1 in a dose-dependent manner, while no inhibition was observed with γ2-pC (residues 219–248) (Figs. 3A and 4A). Inhibition studies with smaller peptides, pN-1, -2, and -3, demonstrated that only pN-3 was active in competing with fibulin-2 binding to γ2-r1 (Fig. 4A, lanes 6–9). The inhibitory activities of synthetic peptides with incremental 1-amino acid deletions (pN-4 to pN-11) from the C terminus of pN-3 were analyzed (Fig. 4, B and C, lanes 1–5). pN-4 (HASADFSVHKIT) and pN-5 (HASADFSVHKI) showed significant inhibition in fibulin-2 binding. pN-6 (HASADFSVH) was less active than pN-4 and pN-5. The inhibitory activity of pN-6 was also confirmed by increasing the concentration of the peptide from 0.2 mg/ml to 1 mg/ml in the inhibition reaction mixtures (Fig. 4C, lane 13). pN-7 (HASADFSVH) was inactive even at 1 mg/ml (Fig. 4C, lane 12). These results indicate that the 10-amino acid sequence HASADFSVHKIT (residues 197–206) was necessary for the fibulin-2 binding and that Ile-207 was required for full inhibitory activity. To determine the minimum size for the inhibitory activity, another set of N-terminal deletions (pN-12 to pN-15) were prepared and tested. pN-14 (SADFSVHKIT) and pN-15 (ASADFSVHKIT) were active at a concentration of 0.2 mg/ml (Fig. 4C, lanes 8 and 9). Both pN-12 (FSVHKIT) and pN-13 (ADFSVHKIT) showed no activity at 0.2 mg/ml, whereas only pN-13 was active at 1 mg/ml (Fig. 4C, lanes 6, 7, 10, and 11). These results indicate that HAS (residues 197–199) was not essential, but Ser-199 was required for the full binding activity. Heptapeptide pN-16 (ADFSVHK), containing the core sequence for the activity, however, was not active even at 1 mg/ml (Fig. 4C, lane 14), indicating that the 10-amino acid sequence was necessary for full inhibitory activity.
suggesting that either Ile-207 or Ser-199 was required for activity. Furthermore, none of three scrambled peptides containing the pN-4 residues were active (Fig. 4C, lanes 16–18), indicating that the activity depends on the specific sequence of amino acids and not on the amino acid composition.

Analysis with the truncated peptides described above demonstrated that the 9-amino acid sequence, SADFSVHKI (residues 199–207), of the γ2 chain was necessary for fibulin-2 binding. We introduced single amino acid substitutions (Fig. 4D, left panel) in this 9-amino acid peptide (pN-21) to identify residues important for the activity. pN-21A1 and pN-21A5 completely abolished the activity, indicating that Phe-202 and Lys-206, because pN-21A3 and pN-21A6 were more active than pN-21A1 and pN-21A5 (Fig. 4D, lanes 5 and 7). The significant loss of activity in pN-21A6 is consistent with a loss observed with pN-6 containing the deletion of Ile-207 of pN-5 (Fig. 4B). The fact that pN-21A2 and pN-21A4 peptides were active in binding suggests that Ser-203 and His-205 were not essential for binding (Fig. 4D, left panel). The change of Phe-202 to Leu (pN-21L1) completely abolished binding (Fig. 4D, right panel, lanes 2 and 3). Although a leucine substitution at Val-204 (pN-21L2) did not decrease its activity, a threonine substitution at Val-204 (pN-21T) abolished its activity (Fig. 4D, right panel, lanes 4 and 6). Furthermore, a glycine substitution at Asp-201 (pN-21G) did not reduce the activity, indicating that this residue was not essential for activity (Fig. 4D, right panel, lane 5). Taken together, these results demonstrate that the nonapeptide 199–207 was the minimum active region with residues Ser, Ala, Phe, Val, Lys, and Ile, critical for the activity. The importance of Ala-200 was not confirmed by a deletion peptide since the peptide was not soluble after deletion of this residue from pN-13 (Table I).

**Fibulin-2 Binds to Native Laminin-5—Binding of fibulin-2 to native laminin-5 was examined by immunoprecipitation assays. The conditioned media from Pam 212 cells were immunoprecipitated with the antibody to the γ2 chain. The precipitates were analyzed by Western blotting with the anti-fibulin-2 antibody. The anti-γ2 chain antibody immunoprecipitated fibulin-2 (Fig. 5A). This co-immunoprecipitation of fibulin-2 was inhibited by peptide pN-4 (Fig. 5B, upper panel). pN-4 did not affect the amount of the γ2 chain immunoprecipitated by the anti-γ2 antibody (Fig. 5B, bottom panel). These results suggest that fibulin-2 binds to native laminin-5 via the pN-4 site in domain IV of the γ2 chain.**

**Fibulin-2 Binds to Laminin-1—** We examined whether fibul-
lin-2 binds to laminin-1 by affinity chromatography. The conditioned media from Pam 212 cell culture were applied on a native laminin-1-coupled Sepharose affinity column. Western blotting of the eluant from this column showed the presence of fibulin-2, indicating that fibulin-2 interacts with laminin-1 (Fig. 6A). Inclusion of 5 mM EDTA to the conditioned media completely eliminated the binding of fibulin-2 to laminin-1, suggesting that this interaction is cation-dependent. Addition of both Ca\(^{2+}\) (8 mM) and Mn\(^{2+}\) (5 mM) to the 5 mM EDTA-containing conditioned media restored binding of the fibulin-2 to laminin-1 (Fig. 6B, upper panel). Mg\(^{2+}\) alone also restored the binding activity, although to a lesser extent. Similar divalent cation dependence was also seen for the binding of fibulin-2 to the recombinant laminin \(\gamma_2\) chain (Fig. 6B, bottom panel). These results indicate that the fibulin-2 binding to laminin-1 and laminin-5 requires divalent cations.

A Fibulin-2 Binding Site of Laminin-1—Peptide pN4 from the \(\gamma_2\) chain had a dose-dependent inhibitory activity for the binding of fibulin-2 to laminin-1 (Fig. 7A). These unexpected observations suggest that laminin-1 binds to fibulin-2 at a site similar to pN4. A protein sequence homology search showed that there are two homologous amino acid stretches in the laminin \(\alpha_1\) chain and one region in each of the \(\alpha_2\), \(\beta_1\), and \(\gamma_2\) chains (Fig. 7B, lower panel). The peptides containing these sequences were analyzed for their inhibitory activity in the binding of fibulin-2 to laminin-1 using the affinity column assay. Peptide p1–654 from the laminin \(\alpha_1\) chain was active (Fig. 7B, left panel, lane 2) and showed a dose-dependent inhibition (Fig. 7B, right panel, lanes 1–5), whereas the other four peptides, p\(\beta_1\)–461, p\(\gamma_2\)–587, p\(\alpha_1\)–279, and p\(\rho_2\)–287, were inactive (Fig. 7B). These results indicate that fibulin-2 binds to laminin-1 through the p\(\alpha_1\)–654 sequence (residues 654–665) of the globular domain IVb of the \(\alpha_1\) chain short arm. In competition assays, pN4 and p\(\rho_1\)–654 showed similar inhibitory activities and blocked binding of fibulin-2 to laminin-1 and to \(\gamma_2\)-r1 (Fig. 7A). p\(\alpha_1\)–654 completely inhibited the binding of fibulin-2 to \(\gamma_2\)-r1 at 0.2 mg/ml comparable to pN4 (Fig. 7B, lanes 7–9). These results suggest that fibulin-2 binds to both laminin-1 and -5 through similar sequence in the \(\alpha_1\) and \(\gamma_2\) chain.

**DISCUSSION**

We have demonstrated through a number of independent methods that fibulin-2 binds to laminin-1 and laminin-5 through the \(\alpha_1\) and \(\gamma_2\) chain short arms, respectively. The binding of fibulin-2 to laminin-5 appears to be a relatively strong interaction, since the anti-\(\gamma_2\) antibody co-immunoprecipitates the complex in the conditioned media. The finding that a synthetic peptide from the \(\gamma_2\) chain could inhibit this complex formation suggests that the fibulin-2 binding site of the \(\gamma_2\) chain is not cryptic and is active in the native laminin-5 molecule. A heptapeptide sequence within laminin \(\gamma_1\) has been delineated for nidogen binding (42–44). As the nonapeptides from the \(\gamma_2\) and \(\alpha_1\) chains inhibit fibulin-2 binding, it is likely that fibulin-2 interacts with a small region in the laminins of similar size to that identified for nidogen. Since the 10 epidermal growth factor-like repeats of fibulin-2 have a calcium-binding motif (21), this region likely contains a site(s) for laminin binding. Consistent with these data is our finding that fibulin-2 binds to both laminin-1 and -5 and this binding is abolished by the addition of EDTA. Recombinant fibulin-2 has been shown to bind strongly to fibronectin in calcium-dependent manner by solid phase radioligand binding assays (23). It also binds to nidogen, although this interaction is only blocked partially by EDTA. However, little binding of fibulin-2 to laminin-1 was found in the solid phase binding assay system. This discrepancy of fibulin-2 binding to laminin-1 may be due to differences in the two assays.

Amino acid truncation and substitution analysis to delineate the region of the \(\gamma_2\) chain responsible for binding to fibulin-2 suggested that residues Ser-199, Phe-202, Val-204, Lys-206, and Ile-207 within the nonapeptide sequence of the \(\gamma_2\) chain (pN21, residues 199–207) were required. Although p\(\beta_1\)–461 from the \(\beta_1\) chain contains similar residues including Phe, Val, and Ile at the positions similar to pN21, it was inactive in inhibiting fibulin-2 binding to laminin-1. The inactive peptide p\(\beta_1\)–461 also contains Leu at the position corresponding to Lys-206 in pN21. This is also consistent with the result that Lys-206 was critical for the activity. The active peptide p\(\alpha_1\)–
binding are F, V, (K/R), and (I/L). The laminin sequences for fibulin-2 binding defined in this report are not present in fibronectin and nidogen. Hence, fibulin-2 may interact with these molecules via different sites (23).

The biological importance of the short arm of the γ2 chain of laminin-5 has been revealed by the finding of a γ2 chain mutation in a human patient with junctional epidermolysis bullosa (7). This patient has an internal deletion of domains III and IV of the γ2 chain short arm, suggesting that the short arm of the γ2 chain is critical for the structural stability of the dermal-epidermal junction. Although the deleted region does not correspond exactly to the site for fibulin-2 binding, it is possible that the deletion perturbs the native conformation of domain IV, resulting in the masking or inactivation of the binding site. Since both molecular abnormalities in laminin-5 and autoantibodies specific to laminin-5 cause blister formation in skin, laminin-5 appears to be important for the integrity of skin. Since fibulin-2 binds to laminin-5, it is possible that it plays a critical role in stabilizing or organizing the epithelial basement membrane during development of skin or wound healing. The recent report that expression of fibulin-2 is markedly increased during skin repair supports this hypothesis (45). It will be of interest to examine whether the active peptide from the γ2 or α1 chain can block formation of basal lamina in an in vitro reconstitution cell culture system (46). It is also interesting to examine whether blistering could be produced by subcutaneous-injection of the active peptide. Further studies will examine the significance of the fibulin-2 binding to laminins.

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