Evidence for Functional Importance of Usherin/Fibronectin Interactions in Retinal Basement Membranes†

Gautam Bhattacharya and Dominic Cosgrove*

Usher Syndrome Center, Boys Town National Research Hospital, 555 North 30th Street, Omaha, Nebraska 68131

Received February 9, 2005; Revised Manuscript Received June 30, 2005

ABSTRACT: Usher syndrome is a genetically heterogeneous disorder characterized by hearing loss with retinitis pigmentosa. Usher syndrome type IIa is the most common of the Usher syndromes, accounting for over half of all cases. The gene encodes a 180 kDa basement membrane glycoprotein called usherin. Here, we demonstrated a specific interaction between usherin and fibronectin in retinal basement membranes. This interaction was confirmed using biochemical, biophysical, and genetic approaches. Surface plasmon resonance assay confirmed that fibronectin binding to usherin is of high affinity and 1:1 stoichiometry. Using a fusion peptide-based co-immunoprecipitation approach, we show that binding to fibronectin occurs at the LE domain of usherin. Recombinant LE domain-specific peptides were engineered that contained single amino acid substitutions corresponding to missense mutations found in humans with Usher syndrome type IIa. Only mutations in loop d of the LE domain abolished the ability of the LE domain to co-immunoprecipitate fibronectin.

Usher syndrome is the leading hereditary cause of combined deafness and blindness in humans. It is an autosomal recessive disease characterized by moderate to severe sensorineural hearing loss and retinitis pigmentosa (RP).[1] The frequency of Usher syndrome has been estimated at 4.4/100000 in the United States (*1*) and 3.0/100000 in Scandinavia (*2*). Usher syndrome is clinically and genetically heterogeneous. To date, three types of Usher syndrome (I, II, III) have been described clinically, and 11 loci and 8 genes have been identified (*3*, *4*). Usher syndrome type II is the most common of the three clinical subtypes (*4*), comprising over half of all cases (*5*). The USH2A gene product, usherin, is a 180 kDa novel basement membrane glycoprotein with four main structural motifs (*6*, *7*). The amino-terminal domain is homologous to the thrombospodin family of extracellular matrix proteins. The thrombospodin-like domains are present in a broad family of proteins. The domains have been shown to function as angiogenesis inhibitors (*8*), MMP-2 and MMP-9 inhibitors (*9*), latent TGF-β1 activators (*10*), and ligands for integrin binding and activation (*11*).

Following the TS-like domain is an LN module. This globular domain is a common feature of laminins, found in five of the known chains (α1, α2, α5, β1, β2), where, like usherin, they are followed by the rodlike laminin-EGF-like modules (LE domains) (*12*, *13*). These domains are required for the polymerization of laminins into the characteristic networks found in basement membranes (*14*, *15*). The LN domain from the laminin α1 chain has been studied extensively and found to bind specifically to integrins $\alpha1\beta1$ and $\alpha2\beta2$ and to the heparin sulfate domains of perlecan (*16*−*18*). The LN module of usherin has the most homology with that of netrin (44% amino acid sequence identity for human). Netrin is viewed as an axonal chemoattractant matrix molecule that plays a role in the guidance of efferent nerve fibers (*19*, *20*). Interestingly, netrin-1 plays a role in axon guidance of the optic nerve (*21*) as well as axon outgrowth from the cochlear nucleus in the brain (*22*).

The LE domain is comprised of a repeat of 60 amino acids containing 8 conserved cysteines (*23*). The LE stands for laminin-type EGF-like domain. All of the known laminin chains, as well as some other matrix molecules including the netrins, contain multiple copies of this structural element, where the domain is present in 3−22 consecutive copies. The arrays of LE domains form rodlike tertiary structures with low flexibility (*13*). The LE domain of the murine laminin γ-1 chain has been shown to bind to nidogen, which is an important structural protein found in basement membranes (*24*). The usherin protein contains 10 repeat units in its LE domain. The b-loop of both the first and fourth LE modules can associate with type IV collagen, an interaction that appears to stabilize usherin in the basement membrane superstructure (*25*). At the carboxy terminus of the usherin protein is three fibronectin type III repeats. These elements are approximately 100 amino acids in length and are a shared domain with at least 45 different families of molecules ranging from cytokine receptors to cell surface binding proteins. The domain is not conserved at the amino acid level but rather is a structural motif where different type III domains may be almost completely dissimilar at the amino acid level and as much as 90% structurally similar (*26*). Like the LE domains, the fibronectin type III domains tend to be present in a tandem series of variable length, forming a series of β-pleated sheet structures. They are known to function as

† Supported by Grants R01 DC04844 and R01 DK55000 to D.C.

* Address correspondence to this author. Phone: (402) 498-6334. Fax: (402) 498-6331. E-mail: cosgrove@boystown.org.

[1] Abbreviations: LE, laminin epidermal growth factor-like domain; TS, thrombospondin-like domain; LN, laminin globular-like domain; FN, fibronectin type III domain; RIPA, radioimmunoprecipitation assay; GST, glutathione *S*-transferase; RP, retinitis pimentosa; RU, resonance units.

10.1021/bi050245u CCC: $30.25
Published on Web 08/04/2005

heparin binding molecules (*27*) as well as integrin binding molecules (*28*).

Very little is known about usherin function in normal tissues. Our laboratory has demonstrated that usherin is a basement membrane protein with a wide, but not ubiquitous, tissue distribution (*7*, *29*). It was found in both vascular and structural basement membranes in many tissues. On the basis of the structural domains, we expect usherin may function through protein/protein interactions with other basement membrane proteins, as well as through binding of receptors on epithelial/endothelial cell surfaces. We previously demonstrated that usherin binds to type IV collagen via specific interaction between the b-loop of the LE domain of usherin and the 7S domain of type IV collagen. In this report, we provide evidence of a second LE-domain-specific usherin interaction. In this case, we show that the d-loop of the LE domain of usherin interacts with fibronectin. This interaction is likely of functional importance, since fusion peptides containing missense mutations in this loop abolish the capacity of these peptides to co-immunoprecipitate fibronectin. Significantly, these same missense mutations we used are ones identified in USH2A patients.

EXPERIMENTAL PROCEDURES

Antibodies and Fusion Peptides. Antibodies used in these studies were raised in rabbits against fusion peptides comprising the entire LN domain of either murine or human usherin and purified on an immunogen-coupled affinity column (Research Genetics, Huntsville, AL). Antibody specificity was validated by Western blotting (*7*). Glutathione *S*-transferase fusion peptides constitute portions of the structural domains of the usherin protein. From the human cDNA, we amplified the first four LE domains and subcloned it into the GST fusion vector, pGEX (Pharmacia Biotech, Piscataway, NJ). The protein was expressed in *Escherichia coli* and purified following the manufacturer's protocols. Missense mutations identified in the human USH2A population were introduced into the wild-type human LE domain fusion peptide construct by amplifying with oligonucleotides containing the desired base substitution. All wild-type and mutant constructs were sequence verified prior to expression. All fusion peptides were collected as soluble protein, not as inclusion bodies, and remain soluble at concentrations up to 15 mg/mL, consistent with an appropriately folded monomer. Comprehensive qualification of the fusion peptides was provided in a previous report (*24*).

Immunohistochemistry. Immunofluorescence immunostaining of murine retinal tissue sections was performed as previously described (*7*) using anti-fibronectin antibody from Biogenesis Inc. (Sandown, NH). Dual immunofluorescence immunostaining was performed using FITC-conjugated anti-rabbit antibodies (against the anti-usherin immunoglobin) and Texas Red-conjugated anti-goat antibodies (against the anti-fibronectin immunoglobulin) purchased from Vector Laboratories (Burlingame, CA). Images were visualized and captured using an Olympus BH-2 fluorescence microscope configured with a Spot-RT digital camera.

Use of Fusion Peptides To Evaluate the Usherin/Fibronectin Interaction. Murine retinal tissues were dissected and homogenized in RIPA (radioimmunoprecipitation assay) lysis buffer (0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing a protease inhibitor cocktail (P8340; Sigma, St. Louis, MO), 0.5 mM dithiothreitol (DTT), and 0.5% phenylmethanesulfonyl fluoride. Homogenized tissues were centrifuged at 11500*g* for 10 min at 4 °C. Supernatants were then incubated with 10 μg of fusion peptides for 4 h at 4 °C with continuous agitation. The reaction mixtures were again incubated with preimmune rabbit serum and a 50% slurry of protein A−Sepharose CL-4B (Sigma, St. Louis, MO) at 4 °C for 1 h to remove nonspecific binding and centrifuged as above for 15 min, and the supernatants were retained as lysates for immunoprecipitation. Either anti-GST (Amersham Biosciences, Piscataway, NJ; used at a 1:1000 dilution) or anti-fibronectin (Biogenesis Inc., Sandown, NH; used at a 1:100 dilution) antisera were added. Samples were incubated overnight at 4 °C and mixed with protein A−Sepharose CL-4B beads on a rocking platform for 1 h at 4 °C. The beads were pelleted by centrifugation as above for 3 min and washed six times with RIPA buffer containing 0.5 M NaCl. Beads were resuspended in gel loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 0.2% SDS, 0.2% bromophenol blue, 20% glycerol), boiled for 3 min, and centrifuged.

Western Blotting. Immunoprecipitated proteins were separated by SDS−PAGE on 12% gels and transferred to 0.45 μm PVDF immobilon P transfer membranes (Sigma, St. Louis, MO). Membranes were quenched at 4 °C overnight in a solution of TBST (Tris-buffered saline plus 0.5% Tween 20; Fisher Scientific, Pittsburgh, PA) and 5% BSA (bovine serum albumin; Sigma, St. Louis, MO) for blocking nonspecific binding. Either primary rabbit polyclonal anti-GST (Amersham Biosciences, Piscataway, NJ,) or anti-fibronectin (Biogenesis Inc., Sandown, NH) diluted 1:1000 in a solution of TBST and 3% BSA was used independently to incubate the blots overnight. After several washes in a solution of TBST, the blots were incubated with a solution of TBST containing an anti-rabbit secondary antibody (horseradish peroxide conjugated; Sigma, St. Louis, MO), diluted 1:20000 for 1 h at room temperature. The blots were then washed several times in TBST, reacted with an ECL (enhanced chemiluminescence kit; Amersham Biosciences Corp., Piscataway, NJ), and exposed to X-ray films.

Expression and Purification of Full-Length Recombinant Usherin. Recombinant usherin was used in the surface plasmon resonance experiments. Protein was expressed in 293 cells and purified as described previously (*24*). Full-length human (GenBank accession no. NM-007123) usherin was assembled and cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) which utilizes the cytomegalovirus promoter to drive expression in eukaryotic cells and a His tag for purification with nickel affinity chromatography. The constructs were transfected into 293 cells, and the clone secreting the most human usherin was selected by Western blot of conditioned culture media. Confluent cells were cultured in DMEM glutamax-1 (Gibco-BRL, Rockville, MD) serum-free medium for 48 h prior to media harvest for purification of recombinant proteins. Conditioned media were concentrated using an Amicon Centricon Plus 80 filter system (Millipore Corp., Bedford, MA) and purified on a nickel affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden). Typical yields of 400 μg of highly purified recombinant usherin per liter of conditioned medium were

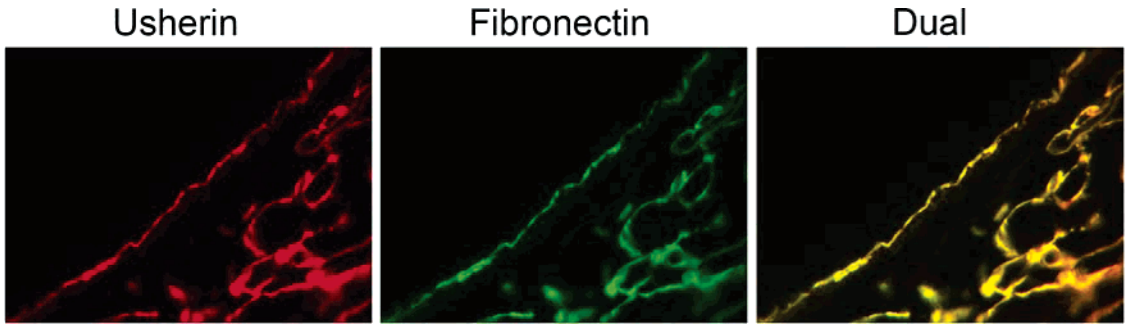

FIGURE 1: Usherin colocalizes with fibronectin in the mouse retina. A cross section of retina from a 7-week-old 129 Sv mouse was subjected to dual immunofluorescence staining using anti-usherin (usherin) and anti-fibronectin (fibronectin) specific antibodies. The right panel (dual) shows that the two proteins colocalize in retinal basement membranes.

attained. Recombinant usherin was aliquoted and stored frozen in PBS at a concentration of 500 μg/mL.

Surface Plasmon Resonance Assay. The BIAcore 1000 upgrade biosensor (Biacore Inc., Piscataway, NJ) was used to measure the binding kinetics of fibronectin to full-length recombinant usherin protein. Usherin was immobilized on a CM5 dextran sensor chip in 10 mM sodium acetate, pH 6.0, using an amine coupling kit (BIAcore Inc.) following the protocol provided by the manufacturer. Purified human fibronectin was purchased from BD Biosciences (Bedford, MA). One hundred fifty resonance units (RU) was coupled to the surface of one flow cell; a second flow cell was activated and blocked and used as the negative control surface. Binding analyses were performed in 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.0005% surfactant P20 at a flow rate of 30 μL/min at 25 °C. Analyses of the proteins were done in the concentration range of 1.5−25 nM, run in duplicate for each sample. The surface was regenerated with 5 μL of 100 mM NaOH at a flow rate of 5 μL/min with no loss of activity. The kinetic rate constants (k_a and k_d) as well as the equilibrium association constant (K_A) and the equilibrium dissociation constant (K_D) were determined using BIAevaluation 3.2RC1 software supplied by the manufacturer where the experimental design correlated with the Langmuir 1:1 interaction model (*30*).

3D Protein Model. All LE domains share common structural elements primarily defined by the eight cysteines. The three-dimensional structure of LE domains in the mouse γ1 chains was used to predict structural consequences of missense mutations. The Molecular Modeling Data Base (*31*) (MMDB) was used to view the model, MMDB no. 6304, PBD entry 1KLO (*33*). The drawing of the structure was generated with the program Cn3D, version 4.1, http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml.

Fibronectin Binding Assay by Mutated Fusion Peptides. One microgram of eye tissue extract was combined in triplicate tubes with 1 μg of each LE domain fusion human usherin peptide in 50 μL of 10 mM phosphate-buffered saline per reaction and incubated at 37 °C for 2 h with gentle shaking. The complexes were immunoprecipitated using anti-GST antibodies (Amersham Biosciences, Piscataway, NJ) as mentioned before using protein A−Sepharose 4B beads (Pharmacia, Piscataway, NJ). The immunoprecipitated material was resuspended in loading buffer, boiled, fractionated by SDS−PAGE (12% gel), and Western blotted using anti-fibronectin antibodies (Biogenesis Inc., Sandown, NH).

Competitive Binding Assay. One microgram of purified fibronectin (Collaborative Biomedical Products, Bedford, MA) was combined with 1 μg of recombinant human usherin in 50 μL of 10 mM phosphate-buffered saline per reaction and incubated at 37 °C for minutes with gentle shaking. Varying amounts of LE domain fusion proteins (0, 1, 10, 30, 100 μg) were added to replicate tubes as competitive inhibitor. After incubating the mixtures for 2 h at 37 °C, the complexes were immunoprecipitated using anti-fibronectin antibodies (Biodesign, Saco, ME) and protein A−Sepharose 4B beads (Pharmacia, Piscataway, NJ). The immunoprecipitated materials were then centrifuged at 13000*g* for 15 min at 5 °C, and the remaining materials were resuspended in loading buffer, boiled, and fractionated by SDS−PAGE (12% gel). The gel was stained with a solution of 0.25% Coomassie Brilliant R-250 and then destained and recorded.

RESULTS

Usherin Colocalizes with Fibronectin in Mouse Retinas. Cryosections of normal mouse eyes were subjected to dual immunofluorescence analysis using antibodies specific for either usherin (in red) or fibronectin (in green). Figure 1 illustrates that when the two images are merged, basement membranes appear yellow, showing that usherin and fibronectin colocalize.

Usherin Binds Fibronectin. We showed that usherin, like most basement membrane proteins, is integrated into basement protein via specific protein interactions, such as type IV collagen (*24*). But interactions with other basement membrane proteins are still unknown. One of the most ubiquitous basement proteins is a network of fibronectin. We employed co-immunoprecipitation experiments to determine whether this interaction was occurring between usherin protein and fibronectin in the basement membrane of the eye. Matrix was extracted from murine eyes (following the removal of the lens). We performed direct immunoprecipitation of the eye extract using antibodies against fibronectin, performed Western blot analysis on the immunoprecipated material, and probed the blot with anti-usherin antibodies. The extract produced a band of correct molecular size (Figure 2, lane A). In another experiment the matrix extract was immunoprecipitated using anti-usherin antibody, immunoblotted, and probed with anti-fibronectin antibodies. The extract produced a band of the correct molecular size for fibronectin (Figure 2, lane B).

The LE Domain of Usherin Contains the Binding Site for Fibronectin. We employed the usherin domain-specific fusion peptides in an attempt to define which domain of usherin interacts with fibronectin. These fusion peptides were

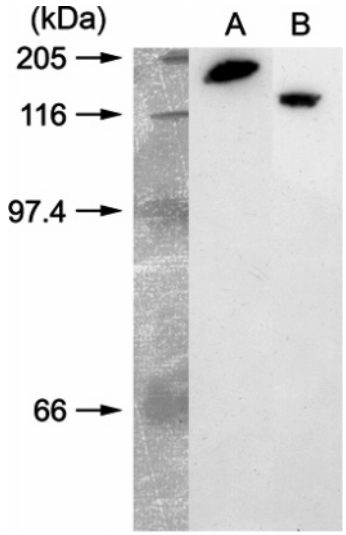

FIGURE 2: The usherin protein interacts with fibronectin. (A) Extracts of matrix from mouse retina were immunoprecipitated with anti-fibronectin antibodies, and the immunoprecipitate was Western blotted using anti-usherin antibodies. (B) Extracts of matrix from mouse retina were immunoprecipitated with anti-usherin antibodies, and the immunoprecipitate was Western blotted using anti-fibronectin antibodies. The molecular mass markers are given in kilodaltons.

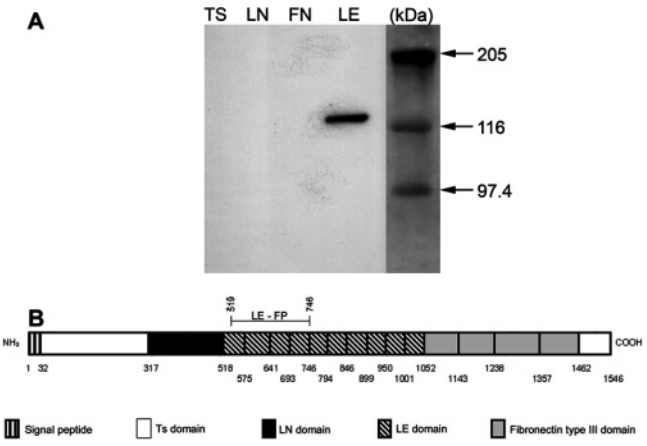

FIGURE 3: The LE domain of usherin interacts with fibronectin. (A) Extracts of matrix from the mouse eye tissues were reacted with the GST fusion peptides consisting of TS, LN, LE, and FN domains and immunoprecipitated with anti-GST antibodies, and the immunoprecipitate was Western blotted using anti-fibronectin antibodies. The molecular mass markers are given in kilodaltons. (B) Schematic showing the structural domains of the usherin protein based on amino acid sequence. The amino acid positions where domains start and end are indicated. The amino acids comprising the LE fusion peptide are also shown.

qualified and characterized for monomeric form and proper folding. This characterization was described in detail in a related article (*24*). The eye matrix extract was reacted with each of the fusion peptides making up the domains of the usherin protein. Complexes were immunoprecipitated using anti-GST antibody (Pharmacia Biotech, Piscataway, NJ), and the immunoprecipitated material was analyzed for fibronectin by Western blotting. The data in Figure 4A illustrate that the fusion peptide constituting the LE domain of usherin formed an immunoprecipitable complex with the predicted molecular size of fibronectin. No other fusion peptide would immunoprecipitate fibronectin, suggesting that the LE domain of usherin contains the binding site for fibronectin.

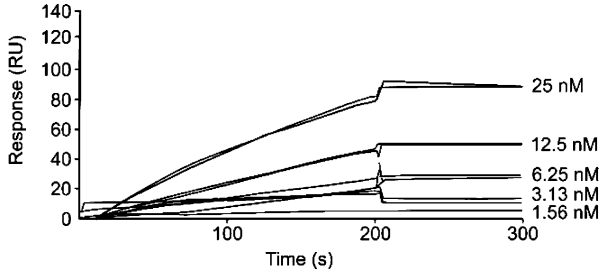

FIGURE 4: Binding of usherin to fibronectin. Binding was analyzed by coupling usherin to a sensor chip and reacting with the indicated concentration range of fibronectin. Response is measured in resonance units (RU).

Figure 4B is provided as a reference showing the structural domains of usherin along with the amino acid composition of the LE domain-specific fusion peptide.

The Usherin/Fibronectin Interaction Is of High Affinity and 1:1 Stoichiometry. The binding kinetics and affinity constants of fibronectin for usherin were measured by surface plasmon resonance using a BIAcore analyzer (Piscataway, NJ). Purified recombinant full-length usherin was covalently coupled to a sensor chip and reacted with varying molar concentrations of purified fibronectin as described in Experimental Procedures. This technique allows the measurement of real time kinetic interactions. The sensogram data in Figure 5 were evaluated to determine the rates of association and dissociation and the affinity constants. The kinetic rate constants for the usherin fibronectin interaction were k_a [1/(M s)] = 1.14×10^5 and k_d (1/s) = 2.92×10^{-5}. The equilibrium affinity constants are K_A (1/M) = 3.91×10^9 and K_D (M) = 2.56×10^{-10}. The data indicate a high-affinity interaction between the two proteins. Interaction data were fitted using the BIA evaluation 3.2 RCI software package (Bia-core Inc.) and found to fit the Langmuir 1:1 model (*30*) with accuracy, which indicates a 1:1 stoichiometry for binding.

Mutations in the d-Loop of the LE Domain of Usherin Abolish FN Binding Activity. Mutation screening of families with usher syndrome type 2A has found a number of missense mutations in the LE domain (*33*−*35*). A number of these missense mutations, which are shown in Figure 3, were engineered into GST expression constructs, and the mutant LE domain fusion peptides were expressed and purified. Eye extract was mixed with the either wild-type or mutant LE domain fusion peptides, and complexes were immunoprecipitated with GST antibodies. Immunoprecipitated material was analyzed by Western blotting using anti-fibronectin antibodies. We found that two out of six mutated LE domain peptides failed to co-immunoprecipitate fibronectin from the tissue extracts (L555V and C620F, Figure 5). Interestingly, both of these missense mutations map to the d-loop of the predicted LE domain structure (Figure 5), strongly suggesting that this loop structure comprises the binding site for fibronectin.

A competitive inhibition assay was devised using purified full-length recombinant usherin and purified human plasma fibronectin and employing either wild-type or mutant fusion peptides as competitive inhibitors of the purified protein. This assay asks whether these peptides, when mixed with the native recombinant proteins at varying molar excess, can compete with fibronectin for binding to usherin. The results

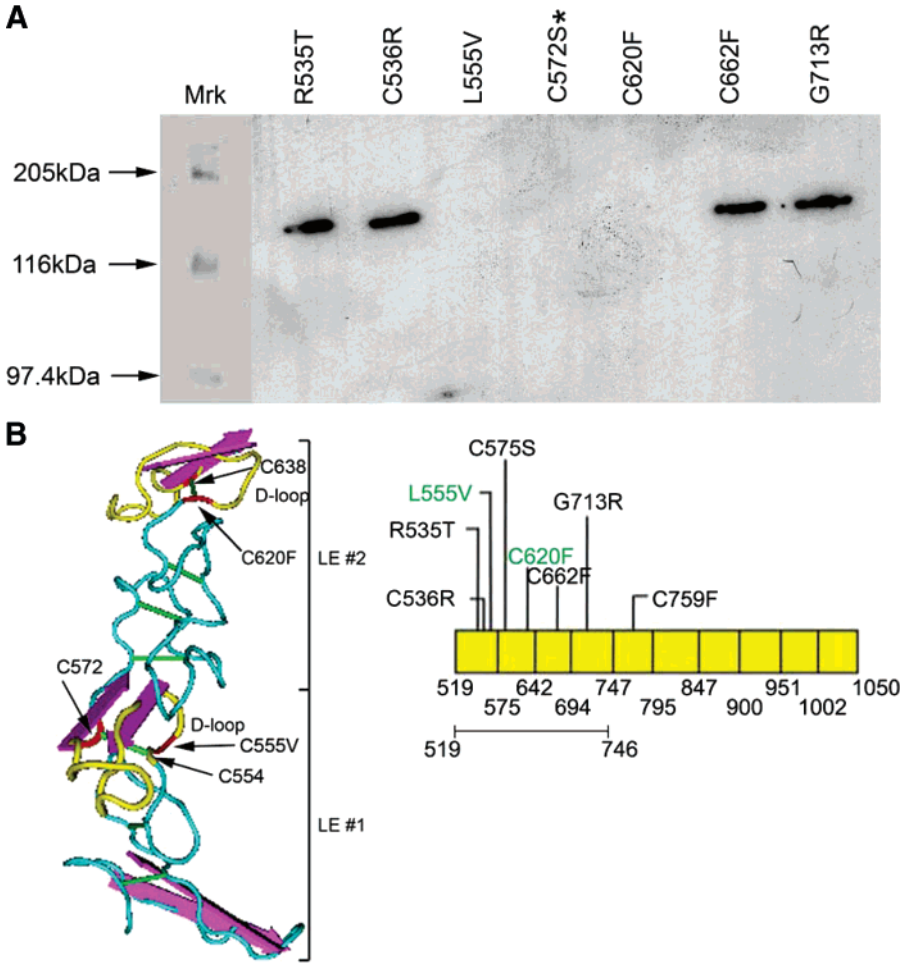

FIGURE 5: Specific missense mutations in the d-loop of the LE domains abolish the fibronectin/collagen interaction. (A) Eye extract (100 μg) and single amino acid substituted recombinant LE domain fusion peptide (1 μg). Following incubation, complexes were immunoprecipitated with anti-GST antibodies, and the immunoprecipitate was Western blotted using anti-fibronectin antibodies. C572S (marked with an asterisk) is the cysteine opposite to C554, which is adjacent to the L555V mutation in the first LE domain of usherin. The C572S mutation has not been found in the human USH2A population. The mutant construct was created to prove the concept that both d-loops are essential for usherin binding to fibronectin. (B) (Left) The three-dimensional structure of the first and second LE modules, based on the mouse laminin γ chain crystal structure. Substituted amino acid residues that obliterate fibronectin binding are shown in red, and disulfide bonds are in green. The d-loops of the two LE domains are in yellow. Amino acid positions of cysteines forming the d-loop structures are indicated. (Right) Location of missense mutations in the LE repeats of usherin. Mutations found in USH2A patients that abolish fibronectin binding are in green.

shown in Figure 6 illustrate that, like with wild-type fusion peptide, the R535T and C536R missense mutant peptides are effective competitors in this assay. In contrast, the L555V and C620F mutant peptides show no evidence of competitive inhibition, even at a 400 molar excess of peptide. These data are entirely consistent with the co-immunoprecipitation (in vivo) data presented in Figure 5.

To further test our hypothesis, we created an additional mutant, C572S (marked with an asterisk). We mutated the cysteine opposite to C554 to validate the model, that integrity of both d-loop structures in LE domains 1 and 2 is imperative for fibronectin interaction. The C572S mutation has not been found in the human USH2A population. The results in Figures 5 and 6 show that this mutant fails to immunoprecipitate fibronectin from retinal extracts (Figure 5) and fails to compete with native usherin for binding to fibronectin in the competitive binding assay (Figure 6). Combined, these data test the prediction that the d-loop structures of LE domains 1 and 2 are essential for fibronectin binding to usherin.

DISCUSSION

Usherin is integrated into the basement membrane via a specific protein interaction of the b-loop of the LE domain of usherin with the 7S domain of type IV collagen (*24*). Here we show that a second basement membrane protein, fibronectin, also interacts with the LE domain of usherin. Fibronectin interacts via the d-loop of the LE domain of usherin. Dual immunofluorescence showed colocalization of usherin/fibronectin in Bruch's membrane underlying the retinal pigment epithelial cells. Co-immunoprecipitation studies using extracts from mouse retinas showed that anti-usherin antibodies co-immunoprecipitate fibronectin and anti-fibronectin antibodies co-immunoprecipitate usherin, showing that the two proteins do indeed directly interact in retinal basement membranes.

We show that at least two missense mutations identified in the human USH2A population in the d-loop (but not the b-loop) disrupt the usherin/fibronectin interaction. This suggests that the usherin/fibronectin interaction is critical for the stability and/or function of usherin in retinal basement

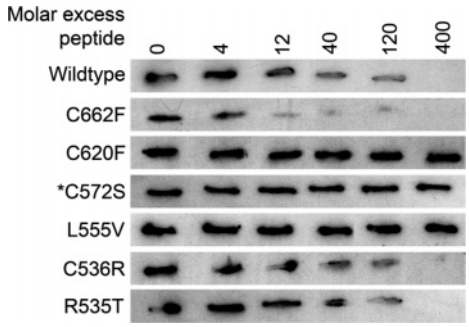

FIGURE 6: Competitive inhibition assay with purified proteins further establishes that specific missense mutations abolish usherin/fibronectin interraction. Purified plasma fibronectin and recombinant human usherin (1 μg of each) were mixed with the indicated relative molar excess amounts of LE domain fusion peptide. Following incubation, complexes were immunoprecipitated with anti-fibronectin antibodies and then immunoprecipitated and fractionated by PAGE. The gel was stained with Coomassie blue, and the lanes corresponding to recombinant usherin were imaged and are shown. C572S (marked with an asterisk), not found in the human population, was included for reasons stated in the legend of Figure 5. Note that L555V, C572S, and C620F lose the capacity to compete with purified fibronectin for binding to usherin, consistent with co-immunoprecipitation data shown in Figure 5.

membranes, since mutations that destroy the interaction result in Usher syndrome type 2a in humans.

It is important to note that the d-loop of the LE domain is structurally distinct from the b-loop, which was found to be the binding site for type IV collagen (*24*). A missense mutation in a critical cysteine residue that abolishes d-loop structure destroys the capacity of the LE domain fusion peptide to co-immunoprecipitate fibronectin (Figure 5) but has no effect on interaction with type IV collagen (*24*). The presence of these nonpolymorphic missense mutations in humans with usher syndrome type IIa but not in phenotypically normal individuals suggests that the d-loop of the LE domain of usherin has important functional properties (*33*, *34*). Further investigation into the functional importance of the usherin/fibronectin interaction is therefore warranted.

It should be noted that the interaction sites for fibronectin with usherin involve both the first and the second LE domains. Two specific missense mutations in these LE domains destroy the capacity of usherin to interact with fibronectin. The Bia-core data predict a 1:1 stoichiometry for binding of fibronectin monomer to usherin (thus, two molecules of usherin bind one disulfide-linked homodimer), suggesting that the binding domain for fibronectin spans both the first and second LE domains, and involves the d-loop of both LE domains. Interestingly, of the two mutations identified that disrupt interaction of usherin with fibronectin, L555V in the first LE domain is one amino acid away from the analogous cysteine (C620F) in the second LE domain, which is also essential for usherin/fibronectin interaction. While the L555V mutation seems to be a conservative amino acid substitution, it has never been found in normal humans but has also been found in autosomal recessive retinitis pigmentosa (*36*), further attesting to the importance of this small change in usherin structure/function. Our engineered peptide containing the C572S mutation (which has not been found in humans with Usher syndrome type IIa) confirms that both d-loops are required for fibronectin interaction (Figures 5 and 6).

Previously documented interactions of fibronectin with basement membrane proteins predominantly suggest a structural role. Fibronectin is known to bind with high affinity to entactin, perlecan, and fibulin-2 (*37*, *38*). Like entactin and perlecan, fibronectin has been purported to play a role in both the assembly of basement membranes and the extracellular matrix, as well as the overall organization of macromolecules within the matrix, which impart its unique biophysical characteristics (*39*−*41*). Our previously documented role for usherin/collagen IV interaction suggests that integration of usherin in the basement membrane is required for stability of usherin (*24*). It is likely that the usherin/fibronectin interaction described in this report is a part of this intermolecular complex involved in the appropriate integration of usherin in retinal basement membranes. Recent evidence suggests that usherin/integrin interactions are central to usherin function in the retina (Bhattacharya et al., submitted for publication). It is becoming increasingly clear that basement membrane architecture is dependent on specific structural properties that dispose the functional domains of basement membrane molecules access to cognate ligands. This specific structural configuration imparts functional specificity and diversity to basement membrane/cell interactions. On the basis of our findings, we propose that usherin/fibronectin interaction is a critical aspect of usherin integration and function in retinal basement membranes.

ACKNOWLEDGMENT

Our sincere thanks to John (Skip) Kennedy for help in the preparation of the figures for publication. Thanks to Dr. Dana Orten for assisting with protein structure data and for helpful comments. We acknowledge the Molecular Interaction Facility at the University of Nebraska Medical Center and the technical expertise of Jody Booth for performing the BIAcore studies.

REFERENCES

1. Boughman, J. A., Vernon, M., and Shaver, K. A. (1983) Usher syndrome: Definition and estimate of prevalence from two high-risk populations, *J. Chronic Dis. 36*, 595−603.
2. Hallgren, B. (1959) Retinitis pigmentosa combined with congenital deafness, with vestibulo-cerebeller ataxia and neural abnormality in a proportion of cases, *Acta Psychiatr. Scand., Suppl. 138*, 1−100.
3. Ahmed, Z. M., Riazuddin, S., Bernstein, S. L., Ahmed, Z., Khan, S., Griffith, A. J., Morell, R. J., Friedman, T. B., Riazuddin, S., and Wilcox, E. R. (2001) Mutations of the protocadherin gene PCDH15 cause Usher syndrome type 1F, *Am. J. Hum. Genet. 69*, 25−34.
4. Petit, C. (2001) Usher syndrome, from genetics to pathogenesis, *Annu. Rev. Genomics Hum. Genet. 2*, 271−297.
5. Rosenberg, T., Haim, M., Hauch, A. M., and Parving, A. (1997) The prevalence of Usher syndrome and other retinal dystrophy-hearing impairment associations, *Clin. Genet. 51*, 314−321.
6. Eudy, J. D., Weston, M. D., Yao, S., Hoover, D. M., Rehm, H. L., Ma-Edmonds, M., Yan, D., Ahmad, L., Cheng, J. J., Ayuso, C., Cremers, C. W. R. J., Davenport, S. L. H., Moller, C. G., Talmadge, C. B., Beisel, K. W., Tamayo, M. L., Morton, C. C., Swaroop, A., Kimberling, W. J., and Sumegi, J. (1998) Mutation of a gene encoding a protein with extracellular matrix motifs in Usher syndrome type IIa, *Science 280*, 1753−1757.
7. Bhattacharya, G., Miller, C., Kimberling, W. J., Jablonski, M. M., and Cosgrove, D. (2002) Localization and expression of usherin: a novel basement membrane protein defective in people with usher syndrime type IIa, *Hear. Res. 163*, 1−11.
8. Nicosia R. F., and Tuszynski, G. P. (1994) Matrix bound thrombospondin promotes angiogenesis in vitro, *J. Cell Biol. 124*, 183−193.

9. Bein, K., and Simons, M. (2000) Thrombospondin type 1 repeats interact with matrix metalloproteinases 2. Regulation of metalloproteinases activity, *J. Biol. Chem. 275*, 32167−32173.
10. Murphy-Ulrich, J. E., and Poezatek, M. (2000) Activation of TGF-beta by thrombospodin-1; Mechanisms and physiology, *Cytokine Growth Factor Rev. 11*, 59−69.
11. Sipes, J. M., Krutzsch, H. C., Lawler, J., and Roberts, D. D. (1999) Cooperation between thrombospodin-1 type repeat peptides and a (v) b (3) integrin ligands to promote melanoma cell spreading and focal adhesion kinase phosphorylation, *J. Biol. Chem. 274*, 22755−22762.
12. Bork, P., Downing, A. K., Kieffer, B., and Cambell, I. D. (1996) Structure and distribution of modules in extracellular proteins, *Q. Rev. Biophys. 29*, 119−167.
13. Beck, K., Hunter, I., and Eugel, J. (1990) Structure and function of laminin; anatomy of a multidomain glycoprotein, *FASEB J. 4*, 148−160.
14. Bruch, M., Landwehr, R., and Eagle, J. (1989) Dissection of laminin by cathespsin G into its long-arm and short-arm structures and localization of regions involved in calcium dependent stabilization and self-association, *Eur. J. Biochem. 185*, 271−279.
15. Yurchenco, P. D., and Cheng, Y. S. (1993) Self-assembly and calcium binding sites in laminin. A three-arm interaction model, *J. Biol. Chem. 268*, 17286−17299.
16. Pfaff, M., Gohring, W., Brown, J. C., and Timpl, R. (1994) Binding of purified collagen receptors a1b1, a2b1, and RGD-dependent integrins to laminins and laminin fragments, *Eur. J. Biochem. 225*, 975−984.
17. Colognato-Pyke, H., O'Rear, J. J., Yamada, Y., Carbonetto, S., Cheng, Y. S., and Yurchenco, P. D. (1995) Mapping of network-forming, heparin-binding, and $\alpha 1\beta 1$ integrin-recognition sites within the a-chain short arm of laminin-1, *J. Biol. Chem, 270*, 9398−9406.
18. Ettner, N., Gobring, W., Sasaki, T., Mann, K., and Timpl, R. (1998) The N-terminal globular domain of the laminin a1 chain binds to a1b1 and a2b1 integrins and to the heparin sulfate-containing domains of perlecan, *FEBS Lett. 430*, 217−221.
19. Leonardo, E. D., Hinck, L., Masu, M., Keino-Masu, K., Fazeli, A., Stoeckli, E. T., Ackerman, S. L., Weinberg, R. A., and Tessier-Lavigne, M. (1997) Guidance of developing axons by netrin-1 and its receptors, *Cold Spring Harbor Symp. Quant. Biol. 62*, 467−478.
20. Metin, C., Deleglise, D., Serafini, T., Kennedy, T. E., and Tessier-Lavigne, M. (1997) A role for netrin-1 in the guidance of cortical efferents, *Development 124*, 5063−5074.
21. Deiner, M. S., Kennedy, T. E., Fazeli, A., Serafini, T., Tessier-Lavigne, M., and Sretavan, D. W. (1997) Netrin −1 and DDC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia, *Neuron 19*, 575−589.
22. Poe, B. H., and Brunso-Bechtold, J. K. (1998) Directed outgrowth from a subset of cochlear nucleus fibers in a collagen-gel matrix, *Brain Res. Dev. Brain Res. 105*, 153−163.
23. Engel, J. (1989) EGF-like domains in extracellular matrix proteins; localized signals for growth and differentiation?, *FEBS Lett. 251*, 1−7.
24. Mayer, U., Poschl, E., Gerecke, D. R., Wagman, D. W., Burgeson, R. E., and Timpl, R. (1995) Low nidogen affinity of laminin-5 can be attributed to two serine residues in EGF-like motif v21114, *FEBS Lett. 365*, 129−132.
25. Bhattacharya, G., Kalluri, R., Orten, D. J., Kimberling, W. J., and Cosgrove, D. (2004) A domain-specific usherin/collagen IV interaction may be required for stable integration into the basement membrane superstructure, *J. Cell Sci. 117*, 233−242.
26. Sharma, A., Askari, J. A., Humphries, M. J., Jones, E. Y., and Stuart, D. I. (1999) Crystal structure of a heparin- and integrin-binding segment of human fibronectin, *EMBO J. 18*, 1468−1479.
27. Barkalow, F. J., and Schwarzbauer, J. E. (1991) Localization of the major heparin binding site in fibronectin, *J. Biol. Chem. 266*, 7812−7818.
28. Bowditch, R. D., Hariharan, M., Tommina, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., and Ginsberg, M. H. (1994) Identification of a novel integrin binding site on fibronectin. Different utilization by b3 integrins, *J. Biol. Chem. 269*, 10856−10863.
29. Pearsall, N., Bhattacharya, G., Wiscarver, J., Adams, J., Cosgrove, D., and Kimberling, W. (2002) Usherin expression is highly conserved in mouse and human tissues, *Hear. Res. 174*, 55−63.
30. Karlsson, R., Michaelsson, A., and Mattsson, L. (1991) Kinetic analysis of monoclonal antibody−antigen interactions with a new biosensor based analytical system, *J. Immunol. Methods 145*, 229−240.
31. Wang, Y., Anderson, J. B., Chen, J., Geer, L. Y., He, S., Hurwitz, D. I., Liebert, C. A., Madej, T., Marchler, G. H., and Marchler-Bauer, A. (2002) MMDB; Enterez's 3D-structure database, *Nucleic Acids Res. 30*, 249−252.
32. Stedefeld, J., Mayer, U., Timpl, R., and Huber, R. (1996) Crystal structure of three consecutive laminin-type epidermal growth factor-like (LE) modules of laminin gamma chain harboring the nidogen binding site, *J. Mol. Biol. 257*, 644−657.
33. Dreyer, B., Tranebjaerg, L., Rosenberg, T., Weston M. D., Kimberling, W. J., and Nilssen, O. (2003) Identification of novel USH2A mutations; implications for the structure of USH2A protein, *Eur. J. Hum. Genet. 8*, 500−506.
34. Weston, M. D., Eudy, J. D., Fujita, S., Yao, S. E., Usami, S., Cremers, C., Greenburg, J., Ramesar, R., Martini, A., Moller, C., Smith, R. J., Sumegi, J., and Kimberling W. J. (2000) Genomic structure and identification of novel mutations in usherin, the gene responsible for usher syndrome type IIa, *Am. J. Hum. Genet. 66*, 1199−1210.
35. Leroy, B. P., Aragon-Martin, J. A., Weston, M. D., Bessant, D. A., Willis, C., Webster, A. R., Bird, A. C., Kimberling, W. J., Payne, A. M., and Bhattacharya, S. S. (2001) Spectrum pf mutations in USH2A in British patient with Usher syndrome type II, *Exp. Eye Res. 72*, 503−509.
36. Seyedahmadi, B. J., Rivolta, C., Keene, J. A., Berson, E. L., and Dryja, T. P. (2004) Comprehensive screening of the USH2A gene in usher syndrome type II and non-syndromic recessive retinitis pigmentosa, *Exp. Eye Res. 79*, 167−73.
37. Hopf, M., Gohring, W., Kohfeldt, E., Yamada, Y., and Timpl, R. (1999) Recombinant domain IV of perlecan binds to nidogens, laminin-nidogen complex, fibronectin, fibulin-2 and heparin, *Eur. J. Biochem. 259*, 917−925.
38. Hopf, M., Gohring, W., Mann, K., and Timpl, R. (2001) Mapping of binding sites for nidogens, fibulin-2, fibronectin and heparin to different IG modules of perlecan, *J. Mol. Biol. 311*, 529−541.
39. Schwarzbauer, J. E., and Sechler, J. L. (1999) Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly, *Curr. Opin. Cell Biol. 11*, 622−627.
40. Brown, J. C., Sasaki, T., Gohring, W., Yamada, Y., and Timpl, R. (1997) The C-terminal domain V of perlecan promotes beta1 integrin-mediated cell adhesion, binds heparin, nidogen and fibulin-2 and can be modified by glycosaminoglycans, *Eur. J. Biochem. 250*, 39−46.
41. Couchman, J. R., Austria, M. R., and Woods, A. (1990) Fibronectin-cell interactions, *J. Invest. Dermatol. 94*, 7S−14S.

BI050245U