

# A domain-specific usherin/collagen IV interaction may be required for stable integration into the basement membrane superstructure

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## Summary

Usherin is a basement membrane protein encoded by the gene associated with Usher syndrome type IIa, the most common deaf/blind disorder. This report demonstrates a specific interaction between type IV collagen and usherin in the basement membrane, with a 1:1 stoichiometry for binding. Genetic and biochemical approaches were used to explore the role of type IV collagen binding in usherin function. We demonstrate binding occurs between the LE domain of usherin and the 7S domain of type IV collagen. A purified fusion peptide comprising the first four LE modules was shown to compete with full-length recombinant usherin for type IV collagen binding. However, synonymous fusion peptides with single amino acid substitutions resulting from missense mutations that were known to cause Usher syndrome type IIa in humans,

failed to compete. Only mutations in loop b of the LE domain abolished binding activity. Co-immunoprecipitation and western blot analysis of testicular basement membranes from the Alport mouse model show a 70% reduction in type IV collagen is associated with a similar reduction in usherin, suggesting the usherin/collagen (IV) interaction stabilizes usherin in the basement membrane. Thus, the domain-specific interaction between usherin and type IV collagen appears essential to usherin stability *in vivo*, and loss of this interaction may result in Usher pathology in humans.

Key words: Usherin, Basement membrane, Collagen (IV), Usher syndrome, Protein interaction

## Introduction

Usher syndrome is the leading genetic disorder of combined hearing and vision loss. The main clinical symptoms are retinitis pigmentosa (RP) and hearing loss. The disorder affects some 3/100,000 people in the general population, but approximately 3-6% of the congenitally deaf (Hallgren, 1959; Nuutila, 1979; Boughman et al., 1983).

Usher syndrome type II is the most common of the three clinical subtypes (Petit, 2001). The USH2A gene encodes a novel basement membrane protein (Eudy et al., 1998; Bhattacharya et al., 2002). Nothing is known about usherin function in normal tissues, or how its absence/dysfunction leads to Usher pathologies. There are four main structural motifs in the 180 kDa glycoprotein. On the amino terminus is a domain with homology to the thrombospondin family of extracellular matrix proteins. The thrombospondins were originally identified as potent inhibitors of angiogenesis, accounting for their tumor suppressor activity (Taraboletti et al., 1990; Nicosia et al., 1994). The well-characterized thrombospondin-1 has diverse functional properties, including activation of latent TGF- $\beta$ 1 (Murphy-Ullrich and Poczatek, 2000), integrin ligation and activation (Sipes et al., 1999), induction of cytoskeletal reorganization (Goicoechea et al., 2000), and inhibition of both MMP-2 and MMP-9 (Bein and Simons, 2000).

Following the thrombospondin domain is an LN module. This globular domain is a common feature of laminins, found in six of the known chains ( $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 5,  $\beta$ 1 and  $\beta$ 2), where, like usherin, they are followed by the rod-like laminin-EGF-like modules (LE domains) (Bork et al., 1996; Beck et al., 1990). These domains are required for the polymerization of laminins into the characteristic networks found in basement membranes (Bruch et al., 1989; Yurchenco and Cheng, 1993). The LN domain from the laminin  $\alpha$ 1 chain has been studied extensively and found to bind specifically to integrins  $\alpha$ 1 $\beta$ 1 and  $\alpha$ 2 $\beta$ 1, and to the heparan sulfate domains of perlecan (Pfaff et al., 1994; Colognato-Pyke et al., 1995; Ettner et al., 1998).

The LE domain comprises repeat units of 60 amino acids containing eight conserved cysteines (Engle, 1989). All known laminin chains, as well as other extracellular matrix molecules including netrins, contain multiple copies of this structural element, present as three to 22 consecutive copies. The arrays of LE domains form rod-like tertiary structures with low flexibility (Beck et al., 1990). The LE domain of the murine laminin gamma-1 chain has been shown to bind to nidogen, which is an important structural protein found in basement membranes (Mayer et al., 1995). Usherin contains 10 LE repeat units that may structurally behave as a rigid spacer between the amino- and carboxy-terminal domains of the molecule.

At the carboxyl terminus of the usherin protein are three fibronectin type III repeats. These domains, of approximately 100 amino acids, are shared with at least 45 different families of molecules ranging from cytokine receptors to cell surface binding proteins. Different type III domains may be almost completely dissimilar at the amino acid level and as much as 90% structurally similar (Sharma et al., 1999). Like the LE domains, the fibronectin type III domains tend to be present in a tandem series of variable length, forming a series of beta-pleated sheet structures. They are known to function as heparan binding molecules (Barkalow and Schwarzbauer, 1991) as well as integrin binding molecules (Bowditch et al., 1994).

In recent reports, we show that usherin is a basement membrane protein with a wide, but not ubiquitous, tissue distribution (Bhattacharya et al., 2002; Pearsall et al., 2002). It is present in both vascular and structural basement membranes in many tissues. Here we provide the first evidence relating usherin function with Usher IIa pathology in the human population. We demonstrate that usherin binds to type IV collagen via a specific interaction between the LE domain of usherin and the 7S domain of type IV collagen. By incorporating known missense mutations into the design of peptides used in a competitive binding assay, we demonstrate that amino acid substitutions within a specific loop structure, but not those identified outside this loop structure, destroy the capacity of the LE domain of usherin to bind the 7S domain of type IV collagen. Using a type IV collagen knockout mouse, we show that this interaction may facilitate stable integration of usherin in the basement membrane superstructure.

## Materials and Methods

### Antibodies

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 by Research Genetics (Huntsville, AL). Antibody specificity was validated by western blotting (Bhattacharya et al., 2002).

### Glutathione S-transferase fusion peptides constitute the structural domains of the usherin protein

From the murine or human cDNA, we amplified each usherin domain (TS, LN, LE and FN, see Fig. 2) and sub-cloned them into the GST-fusion vector, pGEX (Pharmacia Biotech, Piscataway NJ). The proteins were expressed in *E. coli* and purified following protocols provided by the manufacturer. The resulting fusion peptides were limited to 45–46 kDa, including the 26 kDa GST portion, because larger products result in significantly smaller protein yields. Missense mutations identified in the human USH2A population were introduced into 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, and not as inclusion bodies, and remained soluble at concentrations up to 15 mg/ml, consistent with appropriately folded monomers.

### Electrophoresis of protein under denaturing or nondenaturing conditions

To determine whether the fusion peptides exist as monomers or multimers, they were subjected to electrophoretic analysis under denaturing or non-denaturing conditions. To denature the peptides

they were boiled with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 0.2% SDS, 0.2% Bromophenol Blue, 20% glycerol) and were then separated electrophoretically by SDS-PAGE. For non-denaturing conditions no DTT or SDS was used in the sample buffer, and no SDS was used in the PAGE gel.

### Assay for free sulfhydryl groups

All of the fusion peptides contain multiple cysteines. Thus, improperly folded peptides would probably contain free sulfhydryls. To assay for free sulfhydryls, samples (5 µg of each peptide) were analyzed using the Thiol and Sulfide Quantitation Kit (T-6060) according to the manufacturer's instructions (Molecular Probes, Eugene Oregon).

### Use of fusion peptides to evaluate the usherin/collagen interaction

Murine tissues were homogenized in RIPA (RadioImmuno-Precipitation Assay lysis buffer) 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% phenylmethylsulfonyl fluoride. Homogenized tissues were centrifuged at 11,500 *g* for 10 minutes at 4°C. Supernatants were then incubated with 10 µg of fusion peptides for 4 hours at 4°C with continuous agitation. The reaction mixtures were again incubated with pre-immune rabbit serum and a 50% slurry of protein A-sepharose CL-4B (Sigma, St. Louis, MO) at 4°C for 1 hour to remove nonspecific binding, centrifuged as above for 15 minutes and the supernatants were retained as lysates for immunoprecipitation. Either anti-GST (Amersham Biosciences, Piscataway, NJ, used at a 1:100 dilution) or anti-collagen IV (Southern Biotechnology, Birmingham, AL, 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 hour at 4°C. The beads were pelleted by centrifugation as above for 3 minutes and washed six times with RIPA buffer containing 0.5 M NaCl. Pellets 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 minutes, and centrifuged. Immunoprecipitated proteins in the supernatants were separated by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes. In those cases where collagenase digestion was performed prior to electrophoresis, precipitated material was resuspended in 0.2 M NaCl, 5 mM CaCl<sub>2</sub>, 50 mM Tris-HCl (pH 7.4), and digested with 10 units of collagenase A (Advance Biofactures, Lynnbrook, New York) at 22°C for 24 hours. The reactions were terminated by boiling with sample buffer and the proteins electrophoretically separated by SDS-PAGE.

### Direct ELISA

As previously described (Kalluri et al., 1997), type IV collagen from Calbiochem (San Diego, CA) or type IV collagen protomer and individual domains (7 S, triple helical and NC1), isolated from bovine kidney (Gunwar et al., 1991; Kalluri et al., 1996) were used to coat ELISA plates at a concentration of 200 ng per well and blocked with 2% BSA in PBS. Usherin domains (TS, FN, LN and LE fusion proteins) were incubated with each type IV collagen domain and usherin domain binding was probed using rabbit anti-GST antibodies and sheep anti-rabbit IgG secondary antibodies conjugated to alkaline phosphatase. The interaction was concentration dependent, but only the maximal optical densities (OD) are provided.

### Expression and purification of full-length recombinant usherin

Full-length cDNAs encoding murine (GenBank accession no. AF

151717.1) or human (GenBank accession no. NM\_007123) usherin were assembled and cloned into the pcDNA 3.1 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 (ATCC), and the clone secreting the most human or murine usherin was selected by western blot of conditioned culture media. Confluent cells were cultured in D-MEM glutamax-1 (Gibco-BRL, Rockville, MD) serum-free medium for 48 hours prior to media harvest for purification of recombinant proteins. Conditioned media was concentrated using an Amicon Centricon Plus 80 filter system (Millipore Corporation, Bedford, Mass.) and purified on a nickel affinity column (Amersham Pharmacia Biotech, Upsala, Sweden). Typical yields of 400 to 600  $\mu\text{g}$  of highly purified recombinant usherin per liter of conditioned medium were attained. Recombinant usherin was aliquoted and stored frozen in PBS at a concentration of 500  $\mu\text{g}/\text{ml}$ .

### Surface plasmon resonance

The BIAcore 1000 upgrade biosensor (Biacore Inc. Piscataway, NJ) was used to measure the binding kinetics of type IV collagen 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) according to the protocol provided by the manufacturer. 150 resonance units (RU) were coupled to the surface of one flowcell, a second flowcell 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 0.005% surfactant P20 at a flow rate of 30  $\mu\text{l}/\text{minute}$  at 25°C. Analyses of the proteins were done in the concentration range of 1.5 to 25 nM, run in duplicate for each sample. The surface was regenerated with 5  $\mu\text{l}$  of 100 mM NaOH at a flow rate of 5  $\mu\text{l}/\text{minute}$  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 evaluated using the BIAevaluation 3.2RC1 software supplied by the manufacturer where the experimental design correlated with the Langmuir 1:1 interaction model (Karlsson et al., 1991).

### Immunohistochemistry

Immunofluorescence and immunoperoxidase immunostaining of murine cochlear tissue sections were performed as previously described (Bhattacharya et al., 2002; Cosgrove et al., 1998), using anti-collagen IV antibodies from Southern Biotechnology (Birmingham, CA). Dual immunofluorescence immunostaining was performed using FITC-conjugated anti-rabbit antibodies (against the anti-usherin immunoglobulin) and Texas Red-conjugated anti-goat antibodies (against the anti-collagen (IV) immunoglobulin) purchased from Vector Laboratories (Burlingame, CA).

### Immuno-gold colocalization studies

Ultrastructural colocalization studies for Usherin and type IV collagen were performed essentially as previously described (Bhattacharya et al., 2002). Testis from 129Sv/J mice were fixed by heart perfusion with 2% paraformaldehyde in PBS, and post-fixed in this same solution overnight. Ultrathin (70 nm) sections were reacted for 4 hours at room temperature with goat anti-collagen IV antibodies (Southern Biotechnology, Birmingham, AL) and rabbit anti-usherin antibodies added simultaneously. After six 10-minute washes in PBS, specimens were reacted for 2 hours at room temperature with 10 nm gold-conjugated anti-rabbit antibodies and 5 nm gold-conjugated anti-goat antibodies (both from Vector Laboratories, Burlingame, CA). Grids were washed as before, air-dried, counterstained with uranyl acetate, and examined using transmission electron microscopy. Twenty

representative fields were examined, and the distance between gold particles measured.

### 3D protein model

All LE-domains share a common structural element primarily defined by the eight cysteines. The three-dimensional structure of LE domains in the mouse  $\gamma 1$  chain was used to predict structural consequences of missense mutations. The Molecular Modeling Data Base (Wang et al., 2002) (MMDB) was used to view the model, MMDB #6304, PBD entry 1KLO (Stetefeld et al., 1996). Drawings of the structure were generated with the program Cn3D, version 4.0, <http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>.

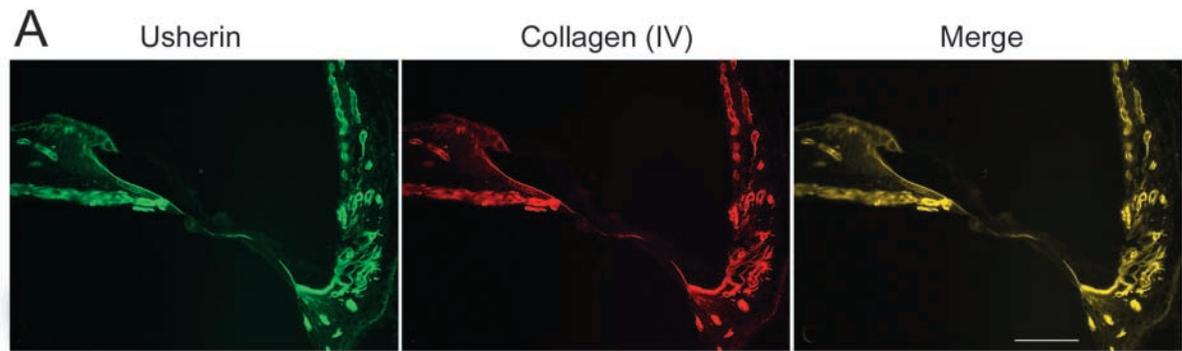
### Analysis of testicular basement membranes

Testes from either normal mice or collagen  $\alpha 3(\text{IV})$ -deficient (Alport) mice were homogenized in 0.05 M Tris-HCl (pH 7.5) buffer and centrifuged at 7000 *g*. The pellet was washed and resuspended in 6 M guanidine-HCl for 24 hours with stirring, centrifuged as above and the supernatant was precipitated with 10 volumes of ethanol. This precipitate was resuspended in PBS for immunoprecipitation. Antisera (either anti-usherin or anti-collagens) was added to 5  $\mu\text{g}$  of extracted protein (quantified by BCA microassay) and incubated overnight at 4°C. Protein A-Sepharose CL-4B beads (15  $\mu\text{m}/\text{ml}$  of a 50% slurry) were added, incubated on a rocking platform for 1 hour at 4°C, pelleted by centrifugation and washed six times with 10 mM NaCl and once with 100 mM NaCl. The protein A-Sepharose 4B Cl was resuspended in gel loading buffer, boiled and centrifuged. Immunoprecipitated protein extract for each sample was electrophoresed into 12% SDS-polyacrylamide gels (SDS-PAGE) and transferred to 0.45  $\mu\text{m}$  PVDF immobilon P transfer membranes (Sigma, St. Louis, MO). Membranes were quenched at 4°C overnight in a solution of TBST (Tris-buffered saline + 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-usherin or collagen type IV (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 peroxidase conjugated; Sigma, St. Louis, MO), diluted 1:20,000 for 1 hour 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.

The intensity of the bands were scanned using the Epicem II instrument (UVP Inc., Upland, CA) and optical densities were determined using Labwork 4.0 software (UVP Inc., Upland, CA). The experiments were performed in triplicate. Significant differences between values were determined by paired *t*-test.

### Competitive binding assay

One microgram of purified type IV collagen (Becton Dickinson, Bedford, MA) was combined with 1  $\mu\text{g}$  of recombinant human usherin in 50  $\mu\text{l}$  of 10 mM phosphate-buffered saline per reaction and incubated at 37°C for 5 minutes with gentle shaking. Varying amounts of LE domain fusion proteins (0, 1, 3, 10, 30, 100  $\mu\text{g}$ ) were added to replicate tubes as competitive inhibitor. After incubating the mixtures for 2 hours at 37°C, the complexes were immunoprecipitated using anti-collagen (IV) antibodies (Bioscience Resource Project, San Diego, CA) and protein A-Sepharose 4B beads (Pharmacia, Piscataway, NJ). The immunoprecipitated materials were resuspended in a buffer composed of 0.2 M NaCl, 5 mM  $\text{CaCl}_2$ , 50 mM Tris-HCl (pH 7.4), and digested with 5 units of collagenase (Worthington, Lakewood, NJ). The samples were then centrifuged at 13,000 *g* for 15 minutes at 5°C, and the remaining



materials resuspended in loading buffer, boiled and fractionated by SDS-PAGE (12% gel). The gel was stained with a solution of a 0.25% Coomassie Brilliant Blue R-250 and then destained and recorded.

## Results

### Usherin colocalizes with type IV collagen

Fig. 1A illustrates dual immunofluorescence immunostaining of a mid-modiolar cross section of the murine cochlea. The cochlea is rich in both vascular and structural basement membranes. Usherin immunostaining is in green, and type IV collagen immunostaining is in red. When the two images are merged, basement membranes appear yellow, illustrating that usherin and type IV collagen colocalize. This was found to be true in all basement membranes where usherin is expressed (Bhattacharya et al., 2002). Ultrastructural colocalization was performed using ultrathin unicryl-embedded tissue sections of testis, which had previously been shown to be rich in usherin (Bhattacharya et al., 2002). Sections were reacted with antibodies specific for both type IV collagen and usherin, then with secondary reagents conjugated to either 5 nm gold particles (specific for the type IV collagen primary antibody) or 10 nm gold particles (specific usherin primary antibody). Based on examination of 20 independent fields, 86% of the time, the two different sized gold particles colocalize (Fig. 1B, arrows), suggesting that the usherin and type IV collagen may directly interact with one another. The gold particles were separated by 23 ( $\pm$ 5) nm. Given these observations, the following study aimed to characterize the nature and function of usherin integration in the basement membrane suprastructure.

### The LE domain of usherin binds specifically to the 7S domain of type IV collagen

It is likely that usherin, like most basement membrane proteins, is integrated into basement membranes via specific protein interactions. We employed a fusion peptide approach as a first step towards examining how usherin is integrated into the basement membrane suprastructure. Fig. 2 illustrates the domain structure of the usherin protein, and indicates the amino acids making up the GST fusion peptides.

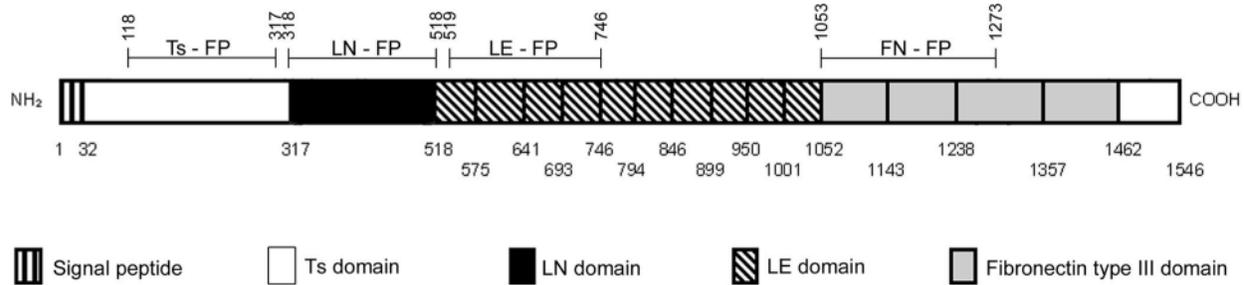
The fusion peptides may not be properly folded. To test this, the peptides were subjected to PAGE analysis under non-denatured and reduced/denatured conditions. Fig. 3A,B shows that the fusion peptides migrate similarly under the two conditions, confirming the absence of multimers. Multimers



**Fig. 1.** Usherin colocalizes with type IV collagen. (A) A mid-modiolar cross section of murine cochlea was subjected to dual immunofluorescence staining using anti-usherin and anti-type IV collagen-specific antibodies. (B) Immunogold colocalization of usherin and type IV collagen. Testis was embedded in Unicryl embedding resin and ultrathin sections were reacted with antibodies specific for either usherin (rabbit anti-usherin) or type IV collagen (goat anti-collagen IV). Sections were then reacted with both 10 nm gold-conjugated anti-rabbit secondary antibodies and 5 nm gold-conjugated anti-goat secondary antibodies. Arrows denote typically observed colocalization for these two antibody probes. Scale bar: A, 50  $\mu$ m; B, 0.2  $\mu$ m.

would be expected if the peptides were improperly folded. The peptide sequences are rich in cysteines. Improper folding would result in a relative absence of disulfide crosslinks. Full-length recombinant usherin (expressed in 293 cells) was assayed for free sulfhydryl groups alongside the fusion peptides under reduced and non-reduced conditions. The results in Fig. 3C show that there are no free sulfhydryl groups in the non-reduced peptides, which supports proper folding. Furthermore, the peptides did not form inclusion bodies in bacteria, and were soluble at 15 mg/ml.

The most abundant and ubiquitous basement membrane protein is a network of type IV collagen heterotrimers composed of  $\alpha$ 1(IV) and  $\alpha$ 2(IV) chains. We employed the usherin domain-specific fusion peptides in an attempt to define whether usherin interacts with type IV collagen in basement membranes. Matrix was extracted from murine cochlea, eye (following the removal of the lens), testis, ovary, liver and heart as described in the methods. The matrix extract was reacted with each of the fusion peptides making up the domains of the usherin protein. Complexes were immunoprecipitated using the anti-GST antibody (Pharmacia Biotech., Piscataway, NJ) and the immunoprecipitated material analyzed for type IV



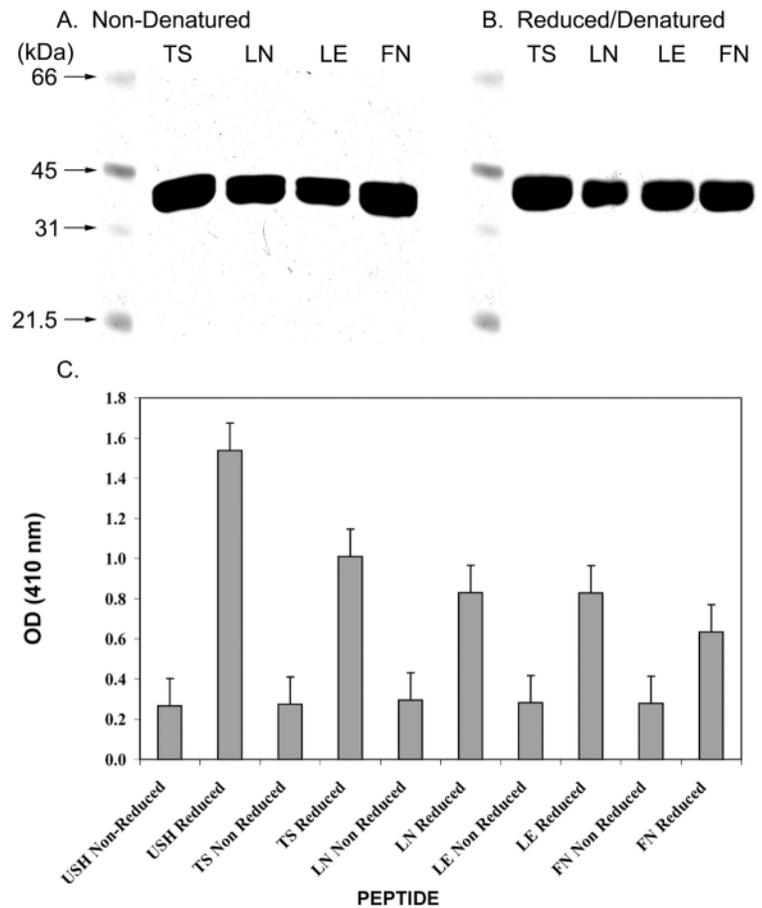
**Fig. 2.** Major structural elements of the usherin protein based on amino acid sequence. The amino acid positions where domains start and end are indicated. The location of peptides used to derive antibodies 1 and 2 used in these studies are shown. Constructs used to generate fusion peptides comprised the indicated portions of the thrombospondin 1, LN, LE and fibronectin type III domains (Ts-FP, LN-FP, LE-FP, and FN-FP, respectively).

collagen by western blotting. The data in Fig. 4A illustrates that the fusion peptide constituting the LE domain of usherin formed an immunoprecipitable complex with type IV collagen in all four tissue extracts. The anti-collagen IV antibodies detected a single band of the molecular size expected for full-length murine collagen  $\alpha 1(IV)$  or  $\alpha 2(IV)$  chains (Saus et al., 1989). The band disappears upon treatment of the immunoprecipitated material with collagenase, further validating that it is indeed type IV collagen. Fusion peptides composing the Ts domain, the LN domain, or the fibronectin type III domain were unable to co-immunoprecipitate type IV collagen (data not shown).

To further verify whether this interaction indeed occurs between these molecules *in vivo*, and is not an anomaly of the fusion peptide system, we performed direct immunoprecipitation of the extracts using antibodies against the type IV collagen  $\alpha 1(IV)$  chain, performed western blot analysis on the immunoprecipitated material and probed the blot with the anti-usherin antibody. All four extracts produced a band of the correct molecular size for usherin (Fig. 4B, about 180 kDa). The band is only detectable in tissues where usherin is expressed, and noticeably absent when extracts from liver and heart are used (Fig. 4B); tissues known to be devoid of usherin (Bhattacharya et al., 2002). Combined, the data in Fig. 4 illustrate that usherin interacts with type IV collagen via the LE domain of the usherin protein.

In an attempt to identify the domain of type IV collagen that interacts with the LE domain, we performed a direct ELISA. Plates were coated with the indicated domains of type IV collagen, which had been prepared from bovine basement membranes as described previously (Kalluri et al., 1997). Coated plates were probed with the fusion peptides, and specific binding was detected using anti-GST antibodies. Fig. 5 shows that binding was only observed for the LE domain fusion peptide. Binding was primarily to the 7S domain of type IV collagen, but some binding was also observed with the helical domain.

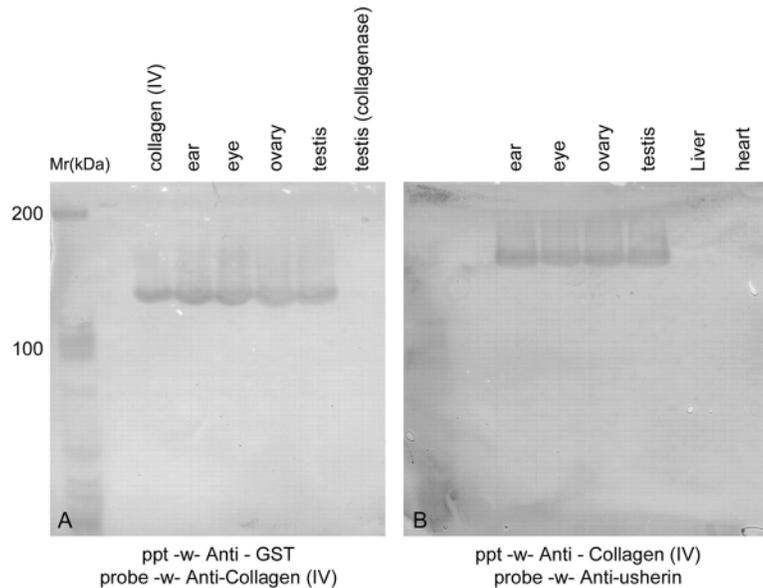
The binding kinetics and affinity constants of type IV collagen for usherin were measured by surface plasmon resonance using a BIA-core analyzer (Piscataway, NJ). Purified



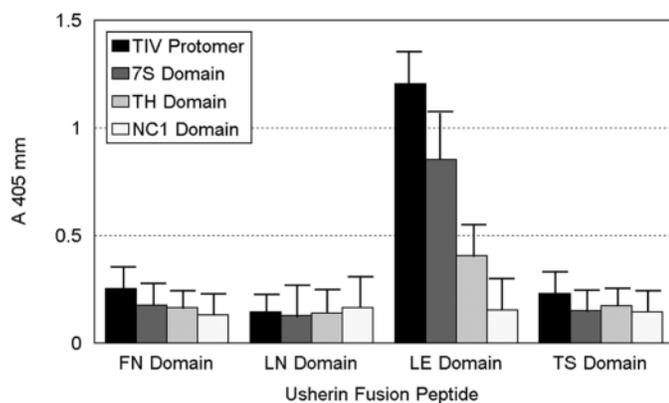
**Fig. 3.** The usherin fusion peptides appear to be properly folded. (A) Usherin fusion peptides were fractionated by PAGE on 15% SDS (denatured) or non-SDS (non-denatured) gels under reducing or non-reducing conditions. (B) Five micrograms of recombinant usherin (ush) or the indicated peptides were analyzed for free sulfhydryl groups under reducing and non-reducing conditions using the Thiol and Sulfide Quantitation Kit (Molecular Probes, Eugene, OR). Free sulfhydryls result in the formation of a colored product with an absorption maxima at 410 nm, which is quantified spectrophotometrically.

recombinant usherin was covalently coupled to a sensor chip and reacted with varying molar concentrations of purified type IV collagen as described in Materials and Methods. This

technique allows the measurement of real-time kinetic interactions. The sensogram data in Fig. 6 were evaluated (see Materials and Methods) to determine rates of association and dissociation and the affinity constants. The kinetic rate constants for the usherin/type IV collagen interaction were:  $k_a/k_d=1.93e^5/6.77e^{-6}$ ; the equilibrium affinity constants were:  $K_A/K_D=1.22e^9/8.19e^{-10}$ . These data indicate a high affinity



**Fig. 4.** The LE domain of usherin interacts with type IV collagen. Extracts of matrix from the indicated mouse tissues were (A) reacted with the fusion peptide consisting of the LE domain, immunoprecipitated with anti-GST antibodies, and the immunoprecipitate western blotted using anti-type IV collagen antibodies, or (B) directly immunoprecipitated with anti-type IV collagen antibodies and the immunoprecipitate western blotted using anti-usherin antibodies. The molecular mass markers are given in kilodaltons.



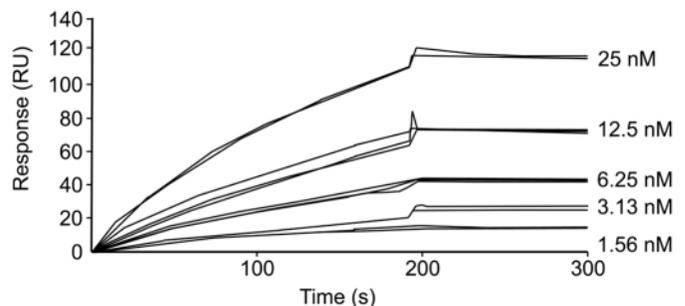
**Fig. 5.** The LE domain of usherin interacts with the 7S domain and the triple helical domain of type IV collagen. Direct ELISA determined interaction between the domain-specific usherin fusion peptides and the indicated domains of type IV collagen. Fusion peptides were reacted with purified collagen domains, which were immobilized on plastic microtiter dishes. Standard deviations are given for triplicate measurements. TIV protomer, the full-length trimeric protomer of type IV collagen; TH, the triple helical domain of type IV collagen; NC1, the non-collagenous domain of type IV collagen.

interaction between the two proteins. Interaction data was fitted using the BIA-evaluation 3.2RC1 software package (BIA-core, Inc.) and found to fit the Langmuir 1:1 model (Karlsson et al., 1991) with high accuracy, which indicates a one to one stoichiometry for binding. Thus there are three usherin binding sites for each heterotrimeric protomer of type IV collagen, and 12 usherin binding sites per 7S domain.

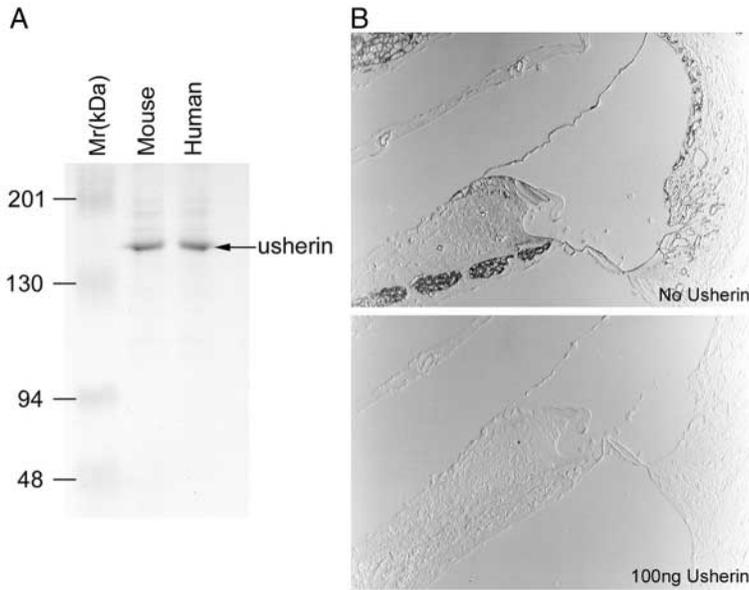
#### Amino acid substitutions resulting from missense mutations in loop b of the LE domain abolish the usherin/collagen IV interaction

Mutation screening of families with Usher syndrome type 2A has revealed a number of missense mutations in the LE modules (Dreyer et al., 2000; Weston et al., 2000; Rivolta et al., 2000; Leroy et al., 2001), almost all of which are in the first 4 LE domains. Assuming the usherin/collagen interaction is important for usherin function, at least some of these missense mutations should influence the type IV collagen binding properties of the LE domain fusion peptide. To test this, we developed a competitive binding assay. Full-length recombinant usherin was expressed in 293 cells and purified from the culture supernatant. Fig. 7A illustrates that the recombinant protein is of high purity and the appropriate molecular size. Fig. 7B shows that the recombinant usherin is capable of competitively inhibiting binding of anti-usherin antibodies to cochlear cryosections, proving that it is indeed usherin. An irrelevant protein did not similarly compete (data not shown). A competitive binding assay was devised where purified human usherin was mixed with an equal mass of type IV collagen, and the LE domain fusion peptide was titrated into the mixture incrementally.

The mixture was immunoprecipitated using anti-collagen (IV) antibodies, the immunoprecipitate digested with collagenase A, and the resulting protein resolved by PAGE followed by Coomassie Blue staining of the gel. The results in Fig. 8A show that the wild-type LE domain is capable of competing with native usherin for binding with type IV collagen. We engineered new fusion peptides that were identical to the wild-type LE domain fusion peptide, except for single amino acid substitutions corresponding to missense mutations identified in the human Usher 2A population. These



**Fig. 6.** Binding of usherin to type IV collagen. Binding was analyzed by coupling usherin to a sensor chip and reacting with the indicated concentration range of type IV collagen. Response is measured in resonance units (RU).



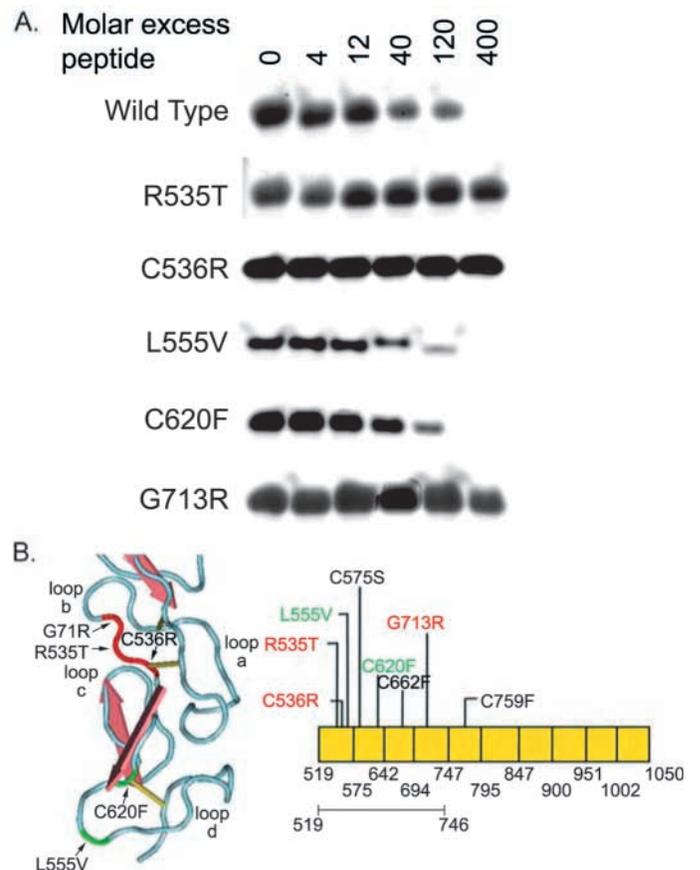
**Fig. 7.** (A) Expression and purification of full-length recombinant human and mouse usherin protein. The full-length cDNAs for human and mouse usherin were cloned into pcDNA 3.1 and transfected into 293 cells. Clones secreting the highest amounts were grown, the supernatant concentrated and recombinant usherin purified by affinity chromatography over nickel columns. Samples of the purified protein were subjected to SDS-PAGE, and the protein stained with Coomassie Blue. The arrow indicates stained protein of the predicted molecular size for usherin (175 kDa). (B) Competitive inhibition of immunostaining using recombinant usherin protein. (Top) Immunostaining with antibody 2 (see Fig. 1). (Bottom) The antibody was mixed with 100 ng of recombinant usherin protein prior to applying it on the tissue section. Absence of staining in B proves that the recombinant protein shown in A is indeed usherin. Both mouse and human usherin competed equally well.

‘mutant’ LE domain fusion peptides were then used in the competitive binding assay. The results in Fig. 8A illustrate that the amino acid substitutions abolished binding activity for three of the five mutations tested. When the mutations were mapped on a 3-dimensional plot of the LE domain protein structure, the three that abolished binding activity mapped to the b loop (Fig. 8B). The two that had no effect on collagen binding mapped to the d loop (one of these mutations, in a conserved cysteine, likely destroys the d loop structure altogether, yet does not affect collagen binding activity). Interestingly, of the mutant peptides that abolished binding activity, the G713R mutation is in the fourth LE domain, while the R535T and the C536R mutations are both in the first LE domain, suggesting that the b loops of multiple LE modules might participate in collagen binding.

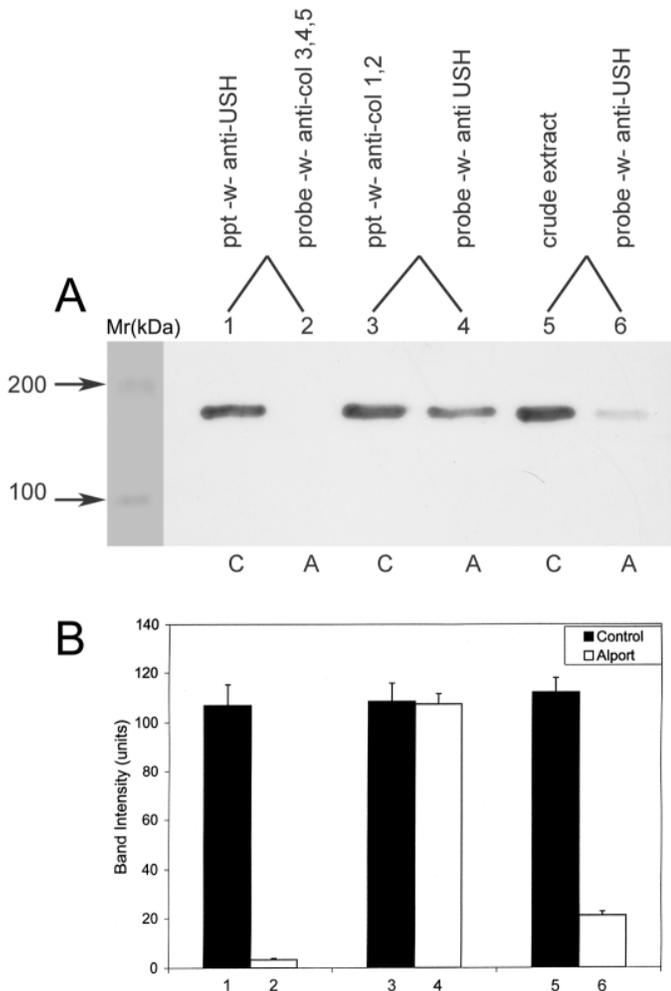
**Collagen binding may stabilize usherin in the basement membrane**

Although the collagen binding studies described above illustrate a strong link between the usherin/collagen interaction and Usher syndrome, they do not tell us why the interaction is important to usherin function. One possibility is that the

interaction stabilizes usherin in the basement membrane. If so, failure to interact would result in a reduction or absence of usherin in the basement membrane. To test this, a mouse model for Alport syndrome was employed. Both type IV collagen and usherin are present in testicular basement membranes (Bhattacharya et al., 2002). In the Alport mouse testis, the loss of type IV collagen  $\alpha$ 3,  $\alpha$ 4 and  $\alpha$ 5 chains reduces the total



**Fig. 8.** Missense mutations in the b loop of the first and fourth LE domains abolish the usherin/collagen interaction. (A) Purified type IV collagen and recombinant human usherin (1  $\mu$ g of each) were mixed with varying relative molar excess amounts of LE domain fusion peptide. Following incubation, complexes were immunoprecipitated with anti-collagen (IV) antibodies, and the immunoprecipitated material digested with collagenase. Remaining material was fractionated by PAGE, and stained with Coomassie Blue. (B) (Left) The three-dimensional structure of a single LE module, based on the mouse laminin  $\gamma$  chain crystal structure. Substituted amino acid residues that obliterate type IV collagen binding activity are shown in red, and residues that did not affect binding are in green. (Right) Location of missense mutations in the LE repeats of usherin. Mutations that abolish collagen binding are in red; mutations that do not affect collagen binding are in green. Mutations in black were not tested.



**Fig. 9.** Usherin binding to type IV collagen may facilitate stable integration of usherin in the basement membrane. (A) Basal lamina was extracted from normal (C) and Alport (A) testes. Solubilized proteins were subjected to immunoprecipitation with anti-usherin antibodies and a western blot probed with anti-type IV collagen  $\alpha$ 3,  $\alpha$ 4 and  $\alpha$ 5 antibodies (lanes 1 and 2); immunoprecipitated with anti-type IV collagen  $\alpha$ 1 and  $\alpha$ 2 antibodies and a western blot probed with anti-usherin antibodies (lanes 3 and 4); or the crude extract analyzed directly (by western blot and probed with anti-usherin antibodies (lanes 5 and 6). Molecular mass markers are in kilodaltons. (B) The experiment in A was run in triplicate using three different sets of animals, and the chemiluminescence signal was quantified and analyzed statistically as described in Materials and Methods. The quantitative data shown in B correspond to the lanes in A.

basement membrane collagen by at least 75% (Kalluri and Cosgrove, 2000). If the stability of usherin in the basement membrane were dependent on the interaction with type IV collagen, we would predict a similar reduction of usherin in Alport testicular basement membranes. Extracts of basal lamina prepared from normal and Alport testis were immunoprecipitated with anti-usherin antibodies. The immunoprecipitate was fractionated by PAGE, and a western blot probed with antibodies specific for type IV collagen  $\alpha$ 3,  $\alpha$ 4 and  $\alpha$ 5 chains. Fig. 9 (lane 1) shows that usherin does interact with these collagen chains, since anti-usherin

antibodies co-immunoprecipitate these chains from normal mouse testicular extracts (Fig. 9, lane 1). No band is observed for the Alport sample (Fig. 9, lane 2), because Alport testicular basement membranes lack these collagen chains (Kalluri and Cosgrove, 2000). When the same extracts were immunoprecipitated with antibodies specific for the type IV collagen  $\alpha$ 1 and  $\alpha$ 2 chains and the blot probed with anti-usherin antibodies, bands of equal intensity are observed for both normal and Alport mice, (Fig. 9, lanes 3 and 4). This is expected, since expression of these collagen chains are unaffected in Alport testicular basement membranes (Kalluri and Cosgrove, 2000). When the extracts are analyzed directly on western blots (not subjected to immunoprecipitation) a marked reduction (80% by scanning densitometry) of usherin is observed in the Alport sample compared with the control sample (Fig. 9, lanes 5 and 6).

## Discussion

Like the many other basement membrane proteins that have been characterized, usherin is expected to integrate into the basement membrane superstructure via specific protein interactions. Here we show that usherin is integrated into the structural architecture of basement membranes via interaction of the LE domain of usherin with the 7S domain of type IV collagen, with a 1:1 stoichiometry for usherin/collagen binding. Furthermore, using a competitive binding assay, we show that missense mutations identified in the human USH2A population in the b loop (but not the d loop) of the first and fourth LE modules disrupt the usherin/type IV collagen interaction. This data supports a crucial role for the usherin/collagen interaction in humans, and illustrates the utility of combining genetic screening for missense mutations with biochemical approaches aimed at defining protein function.

The fact that anti-collagen (IV) antibodies immunoprecipitate the complex from tissue extracts suggests that the interaction is of high affinity, and illustrates that the interaction does indeed occur between native usherin and type IV collagen in basement membranes. Surface plasmon resonance assays confirm high affinity binding of type IV collagen to usherin with a 1:1 stoichiometry. The binding to both 7S domain and triple helical domains suggests that the actual binding site for usherin may be within the hinge region between the 7S domain and triple helical domain. This hinge region is present in the long form of 7S domain as previously reported, and also potentially a part of the triple helical domains as isolated for this present study (Risteli et al., 1980; Dixit et al., 1981). The usherin-collagen (IV) interaction identified in this study probably serves to physically integrate collagen and usherin networks. Type IV collagen has been shown to interact with nidogen and fibulin-1 (Mann et al., 1989; Sasaki et al., 1995), both containing LE domains, however, definitive evidence that type IV collagen is binding to the LE domain has not been described. To our knowledge, this is the first evidence of a binding partner for the 7S domain of type IV collagen in basement membranes.

Missense mutations in the collagen binding domain of usherin, as described here, probably lead to improper assembly of usherin in the basement membrane superstructure. This could result in destabilization of the usherin protein, resulting

in its relative absence, or destabilization of the basement membrane superstructure itself. We show that a reduction of type IV collagen in Alport testicular basement membranes correlates with a similar loss of usherin, suggesting that usherin must be associated with type IV collagen for stable assembly into the basement membrane superstructure. It should be noted, however, that these data do not prove a stabilization mechanism, because alternative mechanisms, such as reduced usherin synthesis, could explain the results. If interaction with collagen (IV) were required for usherin stability, the presence of a missense mutation that prevents collagen binding would have the same net effect as a null mutation (absence of usherin in the basement membranes). Loss of basement membrane proteins as a result of improper assembly is not unprecedented. In Alport syndrome, mutations in a single collagen gene result in the absence of three collagen proteins in the basement membrane because of improper protomer assembly (Kalluri et al., 1993; Gunwar et al., 1998). In laminins, synonymous LE-domain loop structures have been shown to be essential for nidogen binding and stability (Baumgartner et al., 1996; Dreyer et al., 2000). A knock-in mouse was recently described where the nidogen binding domain in the laminin  $\gamma 1$  chain was specifically ablated. Developmental defects in specific basement membranes (of the Wolffian duct and alveolar sacculi) were attributed to ruptures in the basement membrane, indicative of suprastructural destabilization (Willem et al., 2002). These mice showed a relative absence of nidogen 1 in renal basement membranes, suggesting that the interaction is required to stably integrate nidogen 1 in the basement membrane superstructure.

Usherin probably plays a key role in modulating developmental programs of gene expression in the inner ear, since the hearing loss associated with USH2A is congenital. In the retina, however, the pathology is progressive, suggesting a role in maintaining normal cellular homeostasis, possibly via usherin/receptor interactions. The usherin protein contains three canonical domains for which integrin receptor binding has been demonstrated in related molecules; the thrombospondin domain (Sipes et al., 1999), the LN domain (Pfaff et al., 1994; Colognato-Pyke et al., 1995; Ettner et al., 1998) and the fibronectin type III domains (Bowditch et al., 1994). Understanding how usherin influences normal development and functional maintenance of the inner ear and retina will require the identification of the cells and specific receptor interactions involved.

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