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Development of a Functional Skin Matrix Requires Deposition of Collagen V Heterotrimers

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Collagen V is a minor component of the heterotypic I/III/V collagen fibrils and the defective product in most cases of classical Ehlers Danlos syndrome (EDS). The present study was undertaken to elucidate the impact of collagen V mutations on skin development, the most severely affected EDS tissues, using mice harboring a targeted deletion of the α2(V) collagen gene (*Col5a2*). Contrary to the original report, our studies indicate that the *Col5a2* deletion (a.k.a. the *pN* allele) represents a functionally null mutation that affects matrix assembly through a complex sequence of events. First the mutation impairs assembly and/or secretion of the $\alpha1(V)_2\alpha2(V)$ heterotrimer with the result that the α1(V) homotrimer is the predominant species deposited into the matrix. Second, the α1(V) homotrimer is excluded from incorporation into the heterotypic collagen fibrils and this in turn severely impairs matrix organization. Third, the mutant matrix stimulates a compensatory loop by the α1(V) collagen gene that leads to additional deposition of α1(V) homotrimers. These data therefore underscore the importance of the collagen V heterotrimer in dermal fibrillogenesis. Furthermore, reduced thickness of the basement membranes underlying the epidermis and increased apoptosis of the stromal fibroblasts in *pN*/*pN* skin strongly indicate additional roles of collagen V in the development of a functional skin matrix.

The physiological and biomechanical properties of different extracellular matrices depend in large part on the timely and tissue-specific deposition of collagen trimers (or types) that assemble into unique macromolecular aggregates. Among them, the banded fibrils represent the most abundant and widely distributed class of collagen assemblies. Fibril-forming collagens include five distinct types (I to III, V, and XI), which are found in most connective tissues (I, III, and V) or only in cartilage and vitreous (II and XI). Genetic studies have underscored the critical contribution of fibrillar collagens to organismal function by correlating mutations in the α chain subunits with the genesis of several connective tissue disorders (17). Relevant to the present study, they have established the role of collagen V in regulating collagen I fibrillogenesis and in maintaining tissue integrity.

Collagen V is a quantitatively minor component of tissues rich in collagen I, such as dermis, tendons/ligaments, bones, blood vessels, and cornea. Unlike other tissues, where collagen V represents only 1 to 3% of the total collagen fiber content, the relative concentration of this collagen type in cornea is significantly higher, 20 to 25% (3). Collagen V copolymerizes with collagens I and III to form heterotypic I/III/V fibrils in which the triple helical portion of the molecule is embedded and the amino-terminal globular domain projects onto the surface (4, 19). Very thin (5 to 10 nm in diameter) fibrils of collagen V have also been reported immediately near basement membranes and extending into the adjacent interstitial matrix (5, 12, 14, 24). There are several collagen V isoforms that differ in chain composition. They include the most abundant and widely distributed $\alpha1(V)_2\alpha2(V)$ heterotrimer; the α1(V)α2(V)α3(V) isoform found mostly in placenta; and the embryonic $\alpha1(V)_3$ homotrimer. Additionally, heterotypic collagen V/XI trimers have been identified in tissues like bone and vitreous (10). Whereas the functional significance of the isoforms is unknown, the role of collagen V in organizing the corneal matrix is well established.

First, overexpression of a dominant-negative form of the α1(V) chain in chick corneal fibroblasts was shown to decrease collagen V deposition into the matrix and to cause formation of abnormally large collagen I fibrils (22). Second, mice harboring a targeted deletion in the amino-terminal telopeptide of the α2(V) collagen chain (a.k.a. the *pN* mutation) were found to display highly irregular corneal fibrils (1). Altogether, these data corroborated the original hypothesis that the amino-terminal domain of collagen V regulates fibril growth by limiting further accretion of monomers through steric and/or electrostatic hindrance (19). As such, they underscored the importance of both the structural integrity and the expression level of collagen V molecules in conferring optical transparency to the cornea.

Homozygous (*pN*/*pN*) mice die within the first few days of postnatal life, displaying additional manifestations in tissues relatively poor in collagen V, such as skin, tendons/ligaments, bone, and blood vessels (1). The mouse phenotype closely recapitulates the clinical presentation of Elhers-Danlos syndrome (EDS) patients, who carry heterozygous mutations in the α1(V) or α2(V) collagen (COL5A1 and COL5A2) genes

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(21). This heterogeneous heritable connective tissue disorder is characterized by hyperextensible skin, hypermobile joints, bone deformities, fragile vessels, and atrophic scars. Ultrastructural analyses of skin biopsies from some of these EDS patients showed that only 5% of the fibrils present defective ultrastructure (8, 15). Likewise, *Col5a2* mutant mice have revealed that fibril morphology is significantly less affected in dermis than in cornea (1). Although consistent with the relative representation of collagen V in these two tissues, the finding nonetheless contrasts with the severity of the skin phenotype. Moreover, histomorphological anomalies were also noted in the *pN*/*pN* dermis, suggesting that the collagen V mutation may alter also cell-matrix interactions during skin development (1). The present study was therefore undertaken to investigate the precise contribution of collagen V to skin development using the *pN* strain of mutant mice. Our results show that expression of the mutant α2(V) collagen (*Col5a2*) allele triggers an unexpectedly complex sequence of events. They include intracellular degradation of mutant heterotrimers, upregulation of the α1(V) collagen (*Col5a1*) gene, ectopic deposition of α1(V) homotrimers, and assembly of heterotypic fibrils lacking the collagen V component. We also present evidence suggesting that the *pN* mutation perturbs the survival of interstitial fibroblasts and the morphology of the basement membrane at the dermal-epidermal junction. Our data therefore demonstrate for the first time that formation of the $\alpha1(V)_2\alpha2(V)$ heterotrimer is a prerequisite for the assembly of a functional skin matrix.

MATERIALS AND METHODS

Genotyping and RNA analyses. *pN*/+ mice (1) were housed at the Ecole Normale Supérieure Lyon and genotyped by the PCR using primers for *Col5a2* exon 5 sequence (forward primer), and *neo* sequence (reverse primer) or *Col5a2* exon 6 sequence (reverse primer). Total RNA was prepared from wild-type and heterozygous and homozygous mutant mouse tails with the RNeasy kit (QIAGEN) and cultured skin fibroblasts using a published modification of the guanidinium isothiocyanate procedure (7). Reverse transcription (RT) of 1 μg of RNA was performed with Expand reverse transcriptase (Roche). The deleted *Col5A2* gene product was analyzed by RT-PCR amplification using primers specific for exons 5 (5′-GGAATTGATGGAGAGCCAGGTATG-3′) and 7 (5′-GGTCCACGAGAAATTGGAGG-3′). Amplified products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Real-time PCR was performed to quantify the relative abundance of *Col5a1* and *Col5a2* transcripts in wild-type and homozygous skin fibroblasts with gene-specific primers. The assays were carried with a DNA Engine Opticon2 (MJ Research) using intercalation of SYBR green as a fluorescent reporter following the manufacturer's protocol (Quantitect SYBR Green PCR kit; QIAGEN). The primers used were specific for *Col5a1* (forward primer, 5′-GGACTAGTCCGC TTTTCCCTGTCAACTTGTCCGATGG-3′; reverse primer, 5′-GTGGTCACT GCGGCTGAGGAACTTC-3′), *Col5a2* (forward primer, 5′-CAGAAGCCCAG ACGTATCG-3′; reverse primer, 5′-GGTGGTCAGGCACTTCAGAT-3′), and *gapdh* (forward primer, 5′-GTGTTCTACCCCCAATGTG-3′; reverse primer, 5′-AAGTCGCAGGAGACAACCTG-3′). To minimize experimental variations, primers were designed to have similar melting temperatures and GC contents; additionally, all amplified products were of the same size (~100 bp) and did not form dimers. Reactions were performed in triplicate from three separate RNA preparations, and thermal cycling conditions consisted of an initial denaturation step of 94°C for 10 min, 45 cycles of 94°C for 10 s, and annealing and extension at 60°C for 15 s, followed by a final elongation step of 72°C for 5 min. Relative gene expression was determined by using the $2^{-\Delta\Delta CT}$ method as previously described (20). Mean fold changes in *Col5a1* and *Col5a2* gene expression were calculated for wild-type and homozygous samples. Variations were normalized to the relative expression of wild-type samples, and resulting values were plotted as histograms.

Cell cultures and metabolic labeling. Primary skin fibroblasts were grown from skin explants of two 5-day-old wild-type and homozygous *pN*/*pN* mice. Cells were maintained in Dulbecco's modified Eagle's medium-Ham's F-12 medium (Sigma) supplemented with 10% fetal calf serum (Gibco), and antibiotics (Sigma) and 50-μg/ml sodium ascorbate were added to medium, prior to protein and electron microscopy analyses. Subconfluent skin fibroblasts were preincubated in serum-free medium supplemented with sodium ascorbate (50 μg/ml) for 2 h and then labeled with 2-μCi/ml [^{14}C]proline (Amersham Biosciences) overnight. Conditioned media were harvested and centrifuged to remove cell debris. Cell layers were scraped into phosphate-buffered saline (PBS) and centrifuged to pellet insoluble material. Samples were then subjected to pepsin digestion and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6% polyacrylamide) as described below. After electrophoresis, the gels were treated with Amplify solution (Amersham Life Science) to enhance the signal and dried before being exposed to autoradiographic films (Kodak).

Collagen purification and analysis. For collagen purification, skin from 5-day-old wild-type and *pN*/*pN* mice and cell layers of 5-day-cultured wild-type and *pN*/*pN* dermal fibroblasts were washed in cold PBS and digested overnight with pepsin (at an enzyme/substrate ratio of ~1:10) in the presence of 0.2 M NaCl at 4°C. Soluble material and purified collagen V obtained by salt fractionation as previously described were analyzed by SDS-PAGE (5% polyacrylamide) under reducing conditions and stained with Coomassie blue (29). The relative α1(V)/α2(V) ratio was determined with a densitometer (Molecular Dynamics).

Electron microscopy. Skin from 5-day-old wild-type and *pN*/*pN* mice and cell layers from wild-type and *pN*/*pN* skin fibroblasts were fixed at room temperature in 1% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer for 2 h and 30 min, respectively. For immunogold labeling, cultured dermal fibroblasts were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After several washes in PBS, cell layers were digested with hyaluronidase (0.5 mg/ml) for 10 min at 37°C. In some cases, samples were treated with 0.1 M acetic acid for 20 min to partially disrupt fibrils and thus detect collagens buried within them. Cell layers were then incubated with 1% bovine serum albumin in PBS for 30 min and subsequently immersed with collagen I- or collagen V-specific polyclonal antibodies (Novotec, Lyon, France) for 2 h at room temperature, followed by 1 h of incubation with goat anti-rabbit 10-nm-diameter gold conjugate (British BioCell International). This step was followed by overnight fixation in 2% glutaraldehyde in PBS at 4°C. All specimens were postfixed in osmium tetroxide at room temperature for 1 h and embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Philips CM 120 electron microscope (Centre Technique des Microstructures, Université Claude Bernard, Villeurbanne, France). More than 80 fibril diameters from samples corresponding to three independent experiments were measured directly from calibrated electron micrographs. The thickness of basement membrane from skin of two different wild-type and *pN*/*pN* mice was measured in 50 different areas by using the analySIS image analysis program. The resulting values were plotted as histograms.

TUNEL assay. Cultured skin fibroblasts from 5-day-old *pN*/*pN* and wild-type mice were fixed in 4% paraformaldehyde for 1 h, quenched in methanol containing 0.3 to 3% hydrogen peroxide, and treated with 0.1% Triton X-100 in 0.1% sodium citrate. DNA fragmentation was detected with a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay and visualized by using a peroxidase substrate system according to the manufacturer's instructions (POD kit; Roche). Positive controls were incubated with DNase I for 10 min at room temperature.

RESULTS

The *pN* mutation causes disorganization of the dermis matrix. The *pN* allele was engineered to create an in-frame deletion of the exon 6 sequence, which codes for the amino telopeptide of the α2(V) collagen chain. The deleted sequence corresponds to the second noncollagenous (NC2) domain of the protein that is located between the major and minor triple helical regions, COL1 and COL2 respectively, and which is part of the hinge region projecting the NC3 domain outwards (Fig. 1A). As a result of the deletion, the two COL sequences of the mutant α2(V) chain are separated by a single arginine residue (R189) (Fig. 1A). RT-PCR amplification of the wild-type and *pN* transcripts from tail tissue corroborated the DNA genotyping data, in addition to confirming that the two alleles are expressed at comparable levels (Fig. 1B) (1).

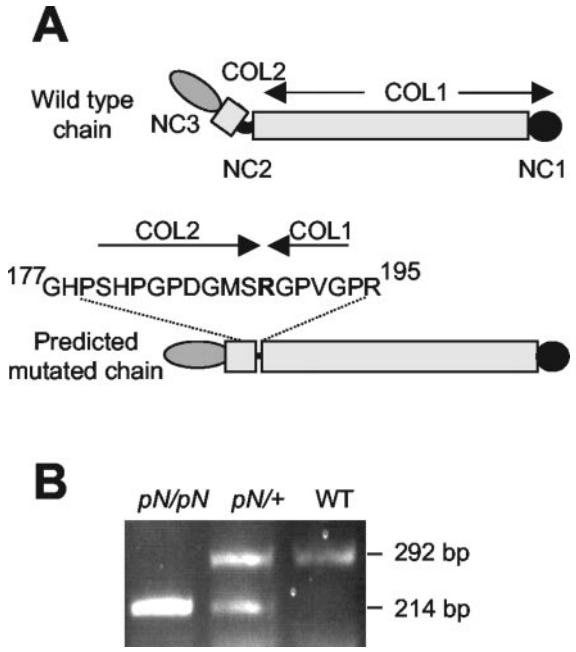

FIG. 1. (A) Schematic illustration of the incidence of exon 6 deletion on the pro-α2(V) chain structure. The exon 6 deletion caused the juxtaposition of the COL1 and COL2 domains. Only an arginine residue from the NC2 domain coded by exon 5 (in boldface) persisted between the two collagenous (COL) domains. NC, noncollagenous domain. (B) Ethidium bromide visualization of RT-PCR products amplified with exon 5 and 7 primers, using total RNA samples purified from wild-type (WT) and heterozygous (*pN*/+) and homozygous (*pN*/*pN*) mouse tails. The size of the smaller product is consistent with the loss of the 78 bp corresponding to exon 6.

Anatomical examination of homozygous mutant mice confirmed the previously described phenotype and in particular, the significant fragility and high stretchability of the skin (1). Because of perinatal lethality, examination of the mutant skin by transmission electron microscopy was limited to two 5-day-old *pN*/*pN* mice. The analysis revealed significant ultrastructural changes in the skin of the mutant compared to the wild-type littermates. Whereas the dermis of wild-type mice contained bundles of tightly packed banded fibrils that completely fill the extracellular space, the *pN*/*pN* dermis showed occasional instances in which the *pN*/*pN* tissue displayed loosely formed fibrils and large areas completely devoid of banded fibrils (Fig. 2A and B). Lacunae filled with proteoglycan aggregates and the characteristic collagen VI beaded filaments were also noted (Fig. 2B).

Dermal fibroblasts from wild-type and mutant mice were isolated and maintained in culture for 5 days in the presence of ascorbate to investigate the effect of the mutation on collagen fibril formation. A significant reduction of the mutant fibroblast cell layer was observed compared to in wild-type fibroblasts (data not shown). In contrast to the compact and dense layer deposited by wild-type cells, *pN*/*pN* fibroblasts produced a thin, transparent, and fragile matrix. These morphological differences matched the ultrastructural analysis of the matrix produced by the cultured dermal fibroblasts. In wild-type cultures, we observed an abundant extracellular matrix composed of numerous banded fibrils that form a tight network with no particular orientation (Fig. 2C). In contrast, *pN*/*pN* fibroblasts produced a sparse network of sinuous and disorganized thin fibrils (Fig. 2D). No periodic striation was detected on the fibrils, probably as a result of the abnormally small diameter of the mutant compared to the wild-type fibrils (17.5 ± 2.8 nm compared to 24.5 ± 3.9 nm). Furthermore, the extracellular space contained abundant dense aggregates resembling proteoglycans (Fig. 2D).

$\alpha1(V)_3$ is the isoform present in *pN*/*pN* skin. A biochemical analysis was undertaken to investigate whether the decrease in fibril number seen in the mutant dermis and in cultured dermal fibroblasts could be accounted for by a defect in collagen V assembly. To this end, metabolic labeling was first performed to investigate the composition of the collagen matrix produced by mutant dermal fibroblasts. Pepsinized [^{14}C]proline-labeled matrix produced by mutant and wild-type fibroblasts were analyzed by gel electrophoresis under reduced and unreduced conditions in order to detect all of the collagen I, III, and V subunits that are present in dermal heterotypic fibrils (Fig. 3A and B). The analysis identified the presence of α1(V) chains, as well as collagen I and III subunits, in both wild-type and *pN*/*pN* samples, but no α2(V) chains in the pepsinized matrix produced by *pN*/*pN* fibroblasts (Fig. 3A and B). To determine whether mutant heterotrimers were normally secreted in the cell medium but not incorporated in the matrix, the pepsinized [^{14}C]proline-labeled conditioned media were also analyzed by gel electrophoresis (Fig. 3C). Collagen I subunits and α1(V) chains were identified in both samples, but no α2(V) chains were observed in *pN*/*pN* fibroblast medium, indicating that mutant heterotrimers were not secreted or were poorly secreted in the medium.

To confirm the absence of α2(V) chain in mutant skin, collagen V was extracted from mutant and wild-type skin and the dermal fibroblast cell layer by pepsin digestion, purified by repeated salt fractionation, and analyzed by SDS-PAGE. As expected, the wild-type controls showed two bands that migrate as the α1(V) and α2(V) chains (Fig. 4A and B). Consistent with the above data, an intense pepsin-resistant band corresponding to the α1(V) chain was also seen in the mutant samples (Fig. 4A and B); however, only a very faint α2(V) band was present in tissue and cell culture samples from *pN*/*pN* mice (Fig. 4A and B). Gel densitometry estimated the ratios between α1(V) and α2(V) chains to be 1.6:1 in wild-type fibroblasts and 15.5:1 in mutant fibroblasts. Collagen electrophoretic patterns often showed the presence of a doublet migrating at around 200 kDa. This corresponds to cross-linked α chain dimers, referred to as β chains. Consistent with the near absence of α2(V) in *pN*/*pN* skin, the band corresponding to the α1(V)/α2(V) dimer was undetectable in mutant samples (Fig. 4A). Overall, the data indicated that the $\alpha1(V)_2\alpha2(V)$ heterotrimer is replaced by the $\alpha1(V)_3$ homotrimer in the *pN*/*pN* skin.

***Col5a1* gene expression is upregulated in *pN*/*pN* fibroblasts.** One possible explanation for our biochemical findings is that the introduction of a targeted mutation in the *Col5a2* gene may reduce gene expression and consequently favor ectopic assembly of the collagen V homotrimer. Alternatively, the mutation may affect formation and/or secretion of the $\alpha1(V)_2\alpha2(V)$ heterotrimer. Although the first possibility is not supported by the RT-PCR data produced here and previously (Fig. 1B) (1), we

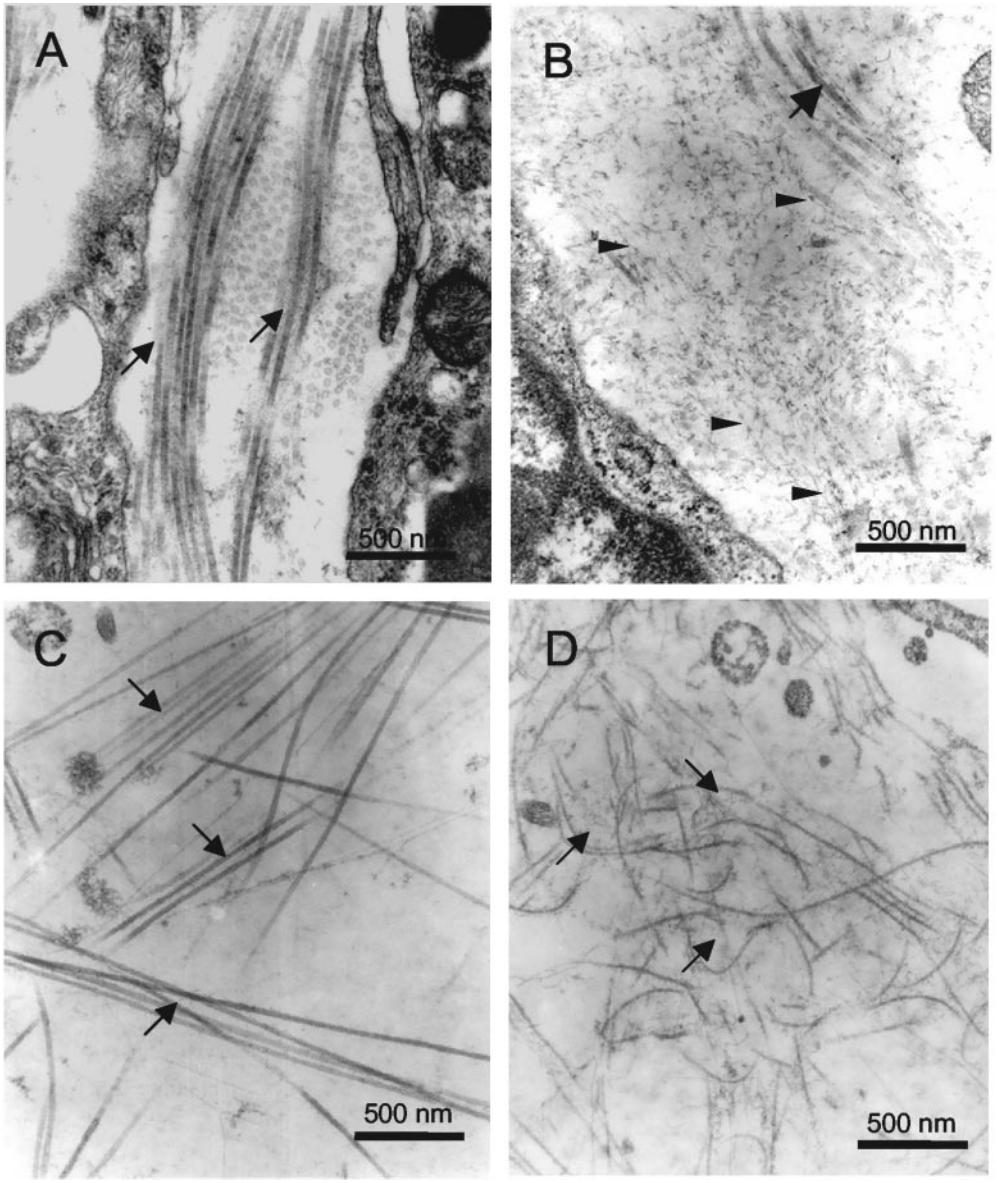

FIG. 2. Ultrastructure of the skin (A and B) and of the extracellular matrix produced by primary dermal fibroblast cultures (C and D). Transmission electron micrographs of cross-sections of dermis show bundles of banded fibrils in the wild-type (A, arrows), whereas only rare fibrils are observed in the mutant (B, arrow). Proteoglycan dense aggregates and collagen VI beaded filaments are observed in the entire extracellular space (B, arrowheads). Cross-sections of the fibril network (arrows) produced by wild-type (C) and *pN*/*pN* (D) primary dermal fibroblasts are shown.

nonetheless reevaluated expression levels of the *Col5a1* and *Col5a2* genes by using the more sensitive technique of real-time PCR. The results of these tests documented that the *Col5a2* gene is expressed at comparable levels in wild-type and *pN*/*pN* mice (Fig. 4C). In contrast, *Col5a1* gene expression in *pN*/*pN* mice was found to be an average of fivefold higher than in wild-type mice (Fig. 4C). Altogether, these results indicate that the *pN* mutation affects both heterotrimer formation and/or secretion and *Col5a1* gene expression.

Heterogeneity of collagen fibrils in *pN*/*pN* dermis. Unlike the $\alpha1(V)_2\alpha(V)$ heterotrimer, in vitro evidence has suggested that $\alpha1(V)$ homotrimers do not participate in the formation of heterotypic I/V fibrils and polymerize into distinctly thin filaments (9). Immunoelectron microscopy using polyclonal antibodies specific to the triple helices of collagens I and V was therefore employed to assess whether or not the collagen V homotrimer participates in the formation of heterotypic fibrils in mutant dermal fibroblast cultures. As expected, the collagen I antibodies decorated *pN*/*pN* thin fibrils in a periodic pattern (Fig. 5A). Collagen V fibrils were readily detected by collagen V antibodies, but the gold particles associated predominantly with loosely formed aggregates distinct from the collagen I fibrils (Fig. 5B and C). Furthermore, acetic acid pretreatment of the samples to unmask epitopes on the triple helical domain of collagen V did not enhance appreciably immunogold labeling (data not shown). These observations suggested that either

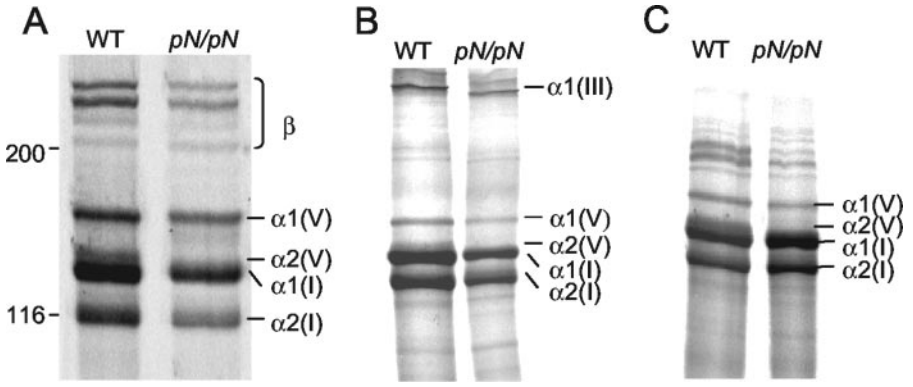

FIG. 3. Collagen composition of the extracellular matrix produced by wild-type (WT) and mutant (*pN/pN*) dermal fibroblast cultures. Cultured fibroblasts were metabolically labeled with [^{14}C]proline. Cell layers (A and B) and cell media (C) were pepsinized and separated by SDS-PAGE (6% polyacrylamide) under reduced (A and C) or unreduced (B) conditions. β forms (brackets) correspond to nonreducible dimers of the different collagen chains. Molecular mass standards are indicated on the left.

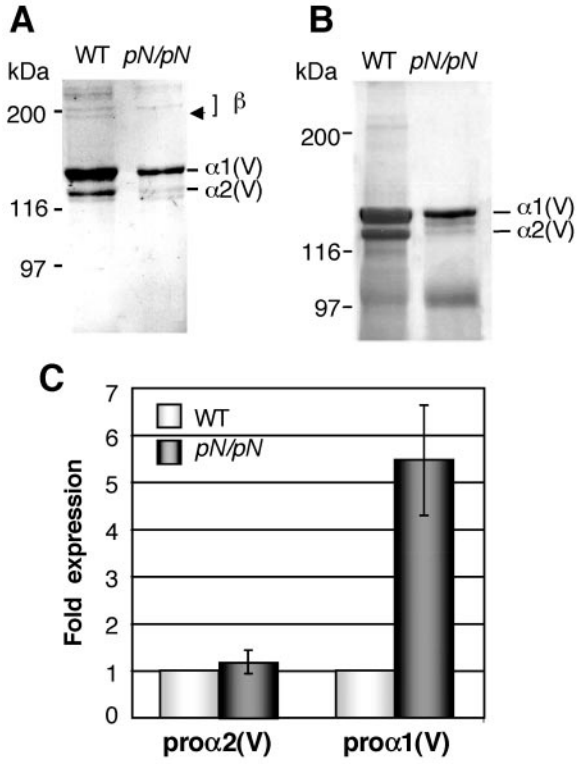

FIG. 4. Prevalence of the collagen V homotrimer in mutant skin. (A and B) Electrophoretic patterns of purified collagen V from skin (A) and from cell layers of dermal fibroblast cultures (B) in wild-type (WT) and mutant (*pN/pN*) mice. Collagens were extracted by pepsin digestion, and collagen V was purified by salt fractionation prior to SDS-PAGE (6% polyacrylamide) separation and Coomassie blue staining. β forms (bracket) correspond to nonreducible α dimers; the lower β form is missing in the *pN/pN* sample (A, arrow). Molecular mass standards are indicated on the left. (C) Expression level of proα2(V) and proα1(V) transcripts in wild-type (WT) and mutant (*pN/pN*) dermal fibroblasts determined by real-time PCR. Expression levels were derived from three independent experiments. Fold expression variations were normalized to the relative expression obtained for wild-type samples.

the heterotypic fibrils are too small to mask collagen V molecules or collagen V is not included into the heterotypic fibrils. We believe that previous in vitro evidence and the distinct immunogold pattern of collagen V antibodies support the latter hypothesis.

***The pN* mutation affects fibroblast survival and basement membrane morphology.** Consistent with the histopathological features previously reported in the *pN/pN* dermis and epidermis, our studies revealed two additional abnormalities seemingly unconnected with fibrillogenesis. First, mutant fibroblast cultures had a significant amount of debris in the medium; in addition, they were abnormally round and detached easily from the substrate (Fig. 6A and B). A TUNEL assay was therefore performed to evaluate the possibility that the mutant cells may undergo apoptosis. In contrast to wild-type cells, a higher percentage of TUNEL-positive cells (15%) were found in *pN/pN* fibroblasts (Fig. 6C and D). Compared to the wild-type control, several *pN/pN* cells also exhibited stereotypical morphological changes characteristic of programmed cell death, such as nuclear chromatin condensation along a particularly dilated nuclear membrane (Fig. 6E and F). Some of the cells displayed more advanced apoptotic features of fragmented nuclei, cell shrinkage, membrane blebbing, abundant vacuoles, and cytoplasm fragmentation (Fig. 6G and H).

The second abnormality of the *pN* mutation was visualized by electron microscopy. Specifically, the ultrastructural analysis revealed that the basement membrane at the dermal-epidermal junction *in* the *pN/pN* mice appears to be thinner and more heterogeneous than that of the wild-type control (Fig. 7A and B). Measurement of the basement membrane thickness confirmed this conclusion by showing that the mutant basement membrane is on the average thinner than the wild-type counterpart (Fig. 7C). In contrast, hemidesmosomes were seemingly normal in mutant and wild-type skin (data not shown).

DISCUSSION

Creation of a mouse strain with a targeted deletion in the α2(V) collagen chain (*pN* mutation) has provided genetic support for the original hypothesis that assembly of corneal fibrils

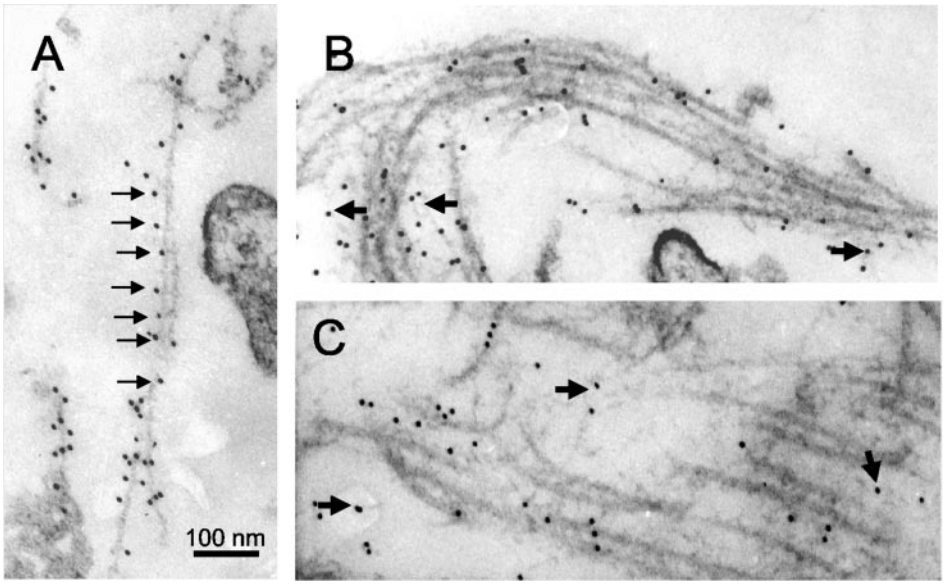

FIG. 5. Localization of collagen I and V in fibrils produced by mutant dermal fibroblast cultures. Electron micrographs show immunogold localization of collagen I (A) and collagen V (B and C). Using collagen I antibodies, gold particles are periodically arranged along the fibrils (A, arrows). Collagen V (B and C) is localized as sparse gold particles along the fibrils and as thin filaments that bound fibrils (arrows).

is an autoregulatory process driven by the minor component of the heterotypic I/III/V collagen fibrils (1). Similar genetic evidence gathered from the characterization of the *cho* mouse independently corroborated this concept by documenting a similar role of collagen XI in modulating collagen II fibrillogenesis in cartilage (18). The *pN/pN* phenotype was also instrumental in predicting that collagen V mutations may be responsible for one of the numerous forms of EDS. This prediction was later confirmed by the identification of several COL5A1 and COL5A2 mutations in patients affected by classical EDS (21, 27). The present study provides new insights into the structural and cellular consequences of the *pN* muta-

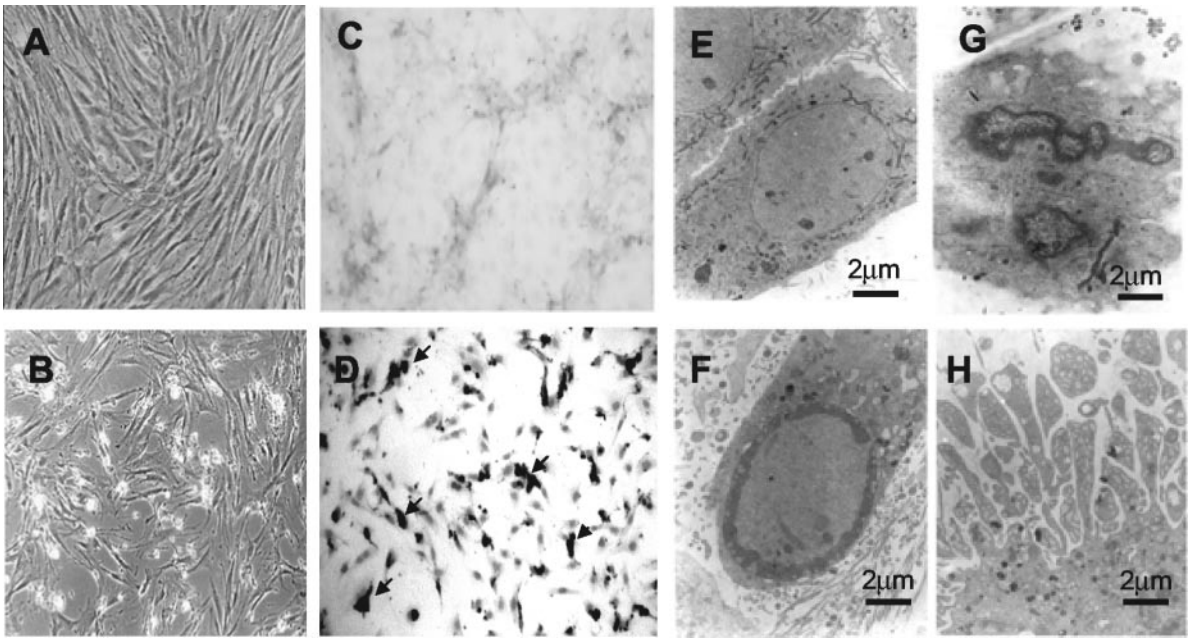

FIG. 6. Morphology of wild-type (A) and mutant (B) cultured dermal fibroblasts. Wild-type fibroblasts (A) reach confluence in a few days, whereas mutant fibroblasts (B) remain dispersed and numerous pieces of debris are present in the medium. (C and D) TUNEL stain of dermal fibroblasts. No staining is observed in wild-type dermal fibroblasts (C), whereas numerous dermal fibroblasts (D, arrows) are TUNEL positive. Magnifications: A and B, ×100; C and D, ×250. (E to H) Transmission electron micrographs of wild-type (E) and *pN/pN* (F to H) dermal fibroblasts. Compare to the wild-type control (E), cells exhibit morphological signs of apopotosis: peripheral chromatin condensation (F), fragmented nuclear morphology (G), and abundance of vacuoles and fragmentation of cytoplasm (H).

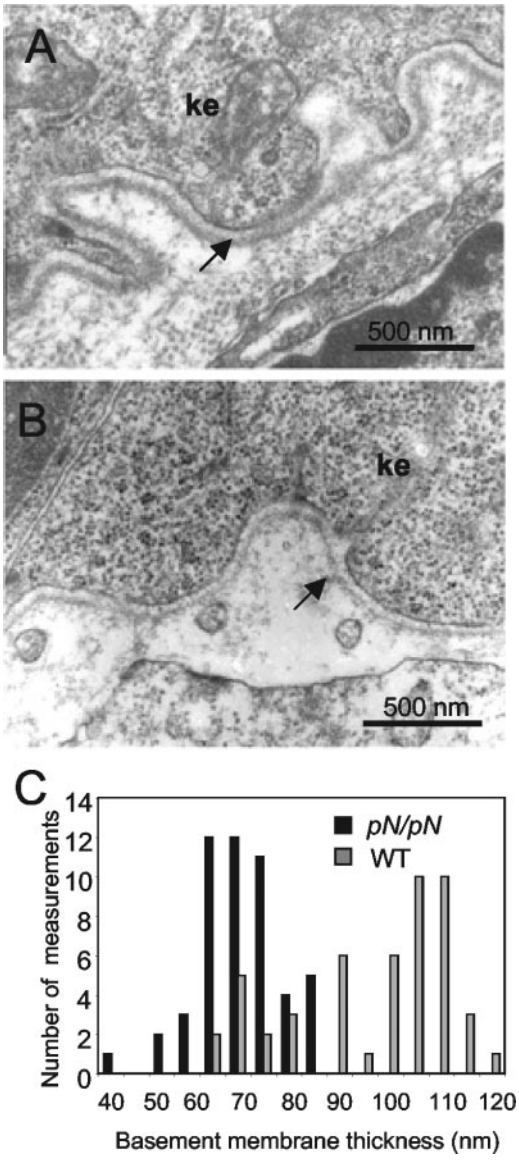

FIG. 7. Cross-sections of the basement membrane (arrows) underlying epidermis from wild-type (A) and *pN*/*pN* (B) mouse skin. ke, keratinocytes. (C) Histograms show basement membrane thickness distributions of wild-type (WT; gray bars) and *pN*/*pN* (black bars) mouse skin.

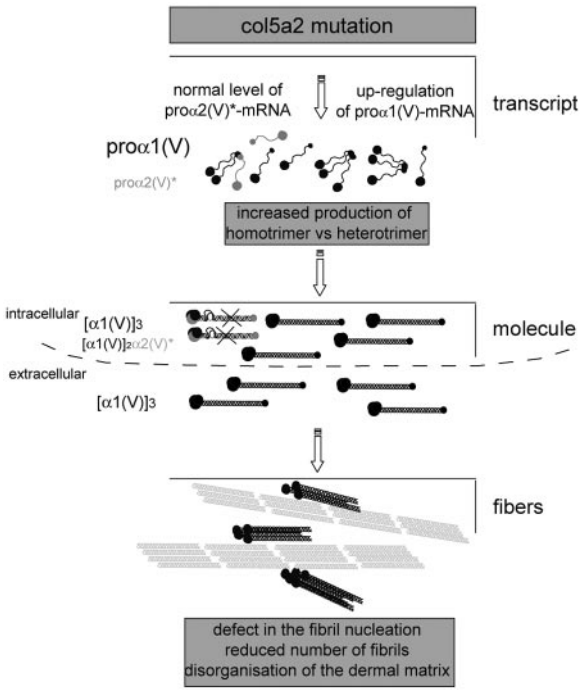

FIG. 8. Theoretical model of the consequences of the *pN* mutation for matrix assembly in skin.

tion on skin development, as well as relevant information about the pathogenic process underlying the most common form of EDS.

Our biochemical data demonstrate that the biosynthetic consequence of the targeted *Col5a2* mutation in mice includes loss of α2(V) chain secretion and deposition coupled with formation of α1(V) homotrimers. Moreover, evidence was presented that accumulation of the α1(V) homotrimer in *pN*/*pN* skin is driven also by the compensatory upregulation of the *Col5a1* gene. These findings are consistent with the notion that homotrimeric association of α2 chains is not possible and with evidence that α1 chains assemble into stable homotrimers in lung cell cultures and in some embryonic tissues (11, 16, 25). Ectopic deposition of α1(V) homotrimers in the developing skin matrix, however, appears unable to compensate for loss of heterotrimers in modulating heterotypic fibril formation. Whereas the original study of the *pN* mouse did not investigate the compensatory loop by *Col5a1*, it did nevertheless examine proα2(V) collagen production by using chain-specific human antibodies (1). Prior, Western analyses did in fact detect a strong collagenase-sensitive band in both wild-type and mutant samples, supporting the conclusion that the *pN* allele was a dominant-negative mutation (1). In light of the biosynthetic and ultrastructural data presented here, this conclusion should be reevaluated.

One possible explanation for our finding is that lack of the NC2 domain in the α2(V) chain and artificial juxtaposition of the two triple helical sequences may impair the registering of the small triple helix and lead to the formation of less stable sites within the amino-terminal extension of the mutant molecule (Fig. 8). In this regard, it is interesting to note that the NC2 domain of the fibrillar collagen I was reported to be involved in conferring thermal stability to the molecule (31). In this scenario, homotrimeric association of α1(V) chains is favored, and the homotrimer is secreted and deposited into the matrix; in contrast, the poorly assembled $\alpha1(V)_2\alpha2(V)$ trimer chain is degraded intracellularly and/or poorly secreted. This latter assumption is reinforced by the lack of detection of stable mutant heterotrimers in cell medium. The fate of the mutant gene product would explain the extreme paucity of α2(V) chains found in the extracellular matrix of the mutant dermis and fibroblast cultures. Furthermore, our evidence suggests that the α2(V)-deficient matrix triggers a regulatory feedback that stimulates upregulation of the *Col5a1* gene, further increasing deposition of $\alpha1(V)_3$ homotrimers that are however precluded from heterotypic fibril formation. Along this line, in

vitro fibrillogenesis experiments showed that, unlike the heterotrimer, recombinant collagen V homotrimers did not form heterotypic fibrils when mixed with collagen I (9). Hence, the *pN* mutation represents a functionally null allele and not a dominant-negative mutation as previously concluded (1).

There are other defects in the *pN* mutant skin that suggest collagen V involvement in processes other than fibrillogenesis. First, the basement membrane of the *pN/pN* epidermis showed reduced thickness compared to the wild type and exhibited imperfections. Such defects were not previously described in *pN/pN* mice or in classical EDS skin biopsies (1, 8, 23, 32). Although collagen V is not a component of basement membrane, these alterations might be related to the presence of thin collagen V filaments in the immediate vicinity of different specialized basement membranes (6, 14, 24). Second, a substantial number of *pN/pN* cells were TUNEL positive and exhibit morphological signs of apoptosis, as judged by electron microscopy. The α2(V)-deficient matrix therefore appears to affect cell survival, conceivably as a result of the loss of important cell-matrix interactions. This conclusion is in line with the established role of the extracellular matrix in sustaining cell survival and with the evidence that loss of adhesion signals causes a form of apoptosis termed anoïkis (13). For example, apoptosis of chondrocytes in mice lacking collagen II has been postulated to be caused by loss of cell-matrix interactions (33). It is also conceivable that collagen V homotrimers may contribute directly to this process, since we have found that the $\alpha1(V)_3$ matrix is less adhesive than the $\alpha1(V)_2\alpha2(V)$ matrix (30; our unpublished data).

An alternative explanation for increased apoptosis is that changes in matrix composition may affect the storage and activation of growth factors, a notion supported by the recent analyses of mice deficient in extracellular matrix microfibrils (28). Specifically, loss of fibrillin 2 has been shown to affect bone morphogenetic protein (BMP)-driven interdigital apoptosis and to result in syndactyly, and apoptosis associated with defective lung septation in fibrillin 1-deficient mice has been causally connected with dysregulation of transforming growth factor β activity (2, 26). Our postulate is further supported by the structural homology between a domain of the α2(V) chain and the cysteine-rich sequence of the amino-propeptide of the collagen IIA that binds transforming growth factor β-1 and BMP-2 (34). It is therefore tempting to speculate that absence of the α2(V) chain in *pN/pN* skin may prevent growth factor sequestration into the matrix and therefore trigger uncontrolled release of these cellular modulators during skin development.

In conclusion, analysis of the *pN/pN* mouse has shed new light on the role of this minor collagen type in skin development and function. Our experiments have documented the critical contribution of the $\alpha1(V)_2\alpha2(V)$ heterotrimer to fibrillogenesis, basement membrane organization, and cell viability. Additionally, this work has elucidated the biosynthetic consequence of the mutation and in so doing redefined the nature of the *pN* allele. Work in progress is examining some of the questions raised by this study, as well as characterizing other affected tissues of the *pN/pN* mice.

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