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Secretion and Assembly of Type IV and VI Collagens Depend on Glycosylation of Hydroxylysines*

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Most lysines in type IV and VI collagens are hydroxylated and glycosylated, but the functions of these unique galactosylhydroxylysyl and glucosylgalactosylhydroxylysyl residues are poorly understood. The formation of glycosylated hydroxylysines is catalyzed by multifunctional lysyl hydroxylase 3 (LH3) *in vivo*, and we have used LH3-manipulated mice and cells as models to study the function of these carbohydrates. These hydroxylysine-linked carbohydrates were shown recently to be indispensable for the formation of basement membranes (Ruotsalainen, H., Sipilä, L., Vapola, M., Sormunen, R., Salo, A. M., Uitto, L., Mercer, D. K., Robins, S. P., Risteli, M., Aszodi, A., Fässler, R., and Myllylä, R. (2006) *J. Cell Sci.* 119, 625–635). Analysis of LH3 knock-out embryos and cells in this work indicated that loss of glycosylated hydroxylysines prevents the intracellular tetramerization of type VI collagen and leads to impaired secretion of type IV and VI collagens. Mice lacking the LH activity of LH3 produced slightly underglycosylated type IV and VI collagens with abnormal distribution. The altered distribution and aggregation of type VI collagen led to similar ultrastructural alterations in muscle to those detected in collagen VI knock-out and some Ullrich congenital muscular dystrophy patients. Our results provide new information about the function of hydroxylysine-linked carbohydrates of collagens, indicating that they play an important role in the secretion, assembly, and distribution of highly glycosylated collagen types.

Collagen biosynthesis contains many co- and post-translational modifications that are unique to collagenous proteins. These modifications include hydroxylation of lysyl residues and their further glycosylation to galactosylhydroxylysyl and glucosylgalactosylhydroxylysyl residues. Lysyl hydroxylase (LH[2]; E.C. 1.14.11.4) catalyzes the hydroxylation of lysyl residues in the *Y*-position of the repeating collagen Gly-*X*-*Y* triplets (1, 2), and three lysyl hydroxylase isoforms have been characterized: LH1, LH2, and LH3 (3–10).

LH3 is a multifunctional enzyme containing, in addition to lysyl hydroxylase activity, collagen galactosyltransferase (E.C. 2.4.1.50) and glucosyltransferase (E.C. 2.4.1.66) activities *in vitro* (11–13). LH3 is functional in both the endoplasmic reticulum and extracellular space (14), and loss of LH3 leads to early embryonic lethality (15, 16). We have reported recently that LH3 is also multifunctional *in vivo* and that especially the glucosyltransferase activity of LH3 is essential for normal embryonic development (16).

The amount of lysine hydroxylation and glycosylation varies between different collagen types. Type IV and VI collagens are highly hydroxylated and glycosylated, whereas fibrillar collagens, especially types I and III, have much lower levels of these modifications (1, 17, 18). The hydroxylysyl residues have an important role in collagen cross-linking (19, 20), but the function of the glycosylation of the hydroxylysyl residues is still poorly understood. The role of glycosylated hydroxylysines in the formation and morphology of collagen fibrils has been debated (21, 22), and we have shown that they are necessary for the correct basement membrane localization of type IV collagen (16).

We produced previously three different mouse lines with a manipulated LH3 gene (16). The mice with no or a very low level of the LH3 protein, thus lacking the lysyl hydroxylase and glucosyltransferase activities of LH3, had an embryonic lethal phenotype. The mouse line (LH mutant) with a point mutation that specifically destroys the LH activity of LH3 without affecting glycosyltransferase activities (11) was viable, but showed ultrastructural alterations (16). In this study, we used LH3 knock-out and LH mutant mice and fibroblasts derived from them as a model to investigate the functions of glycosylated hydroxylysines of collagens. This work provides new information about the function of hydroxylysine-linked carbohydrates of collagens, indicating that they play an important role in the secretion of type IV collagen and in the tetramerization and

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[2] The abbreviations used are: LH, lysyl hydroxylase; PBS, phosphate-buffered saline; EM, electron microscopy.

secretion of type VI collagen and also affect the assembly and distribution of these collagen types.

EXPERIMENTAL PROCEDURES

LH3-manipulated Mice—The production of the LH3 knock-out and LH mutant mice has been described previously (16).

Isolation of Embryonic Mouse Fibroblast and Newborn Mouse Skin Fibroblast Cell Lines—Embryos from heterozygous LH3 knock-out crosses were used to derive embryonic mouse fibroblasts. Embryonic day 8.5 wild-type and LH3 knock-out embryos were dissected, washed with phosphate-buffered saline (PBS), and placed in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 20% fetal calf serum (PromoCell), penicillin and streptomycin, and 50 μg/ml ascorbic acid in 24-well plates. After several days in culture, the cells were trypsinized and transferred to 35- and 100-mm culture dishes, and culture was continued in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin/streptomycin, and 50 μg/ml ascorbic acid. Embryonic mouse fibroblasts became spontaneously immortalized after several weeks in culture. The skin of the homozygous newborn LH mutant and wild-type mice was dissected; sliced; washed with PBS; and placed in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin, and 50 μg/ml ascorbic acid under coverslips. The skin cells were cultured as described above for embryonic mouse fibroblasts. The genotype of the cells was confirmed by PCR as described previously (16).

Immunoblotting—The cells were washed, scraped into homogenization buffer (0.2 M NaCl, 0.1 M glycine, 0.1% Triton X-100, 50 μM dithiothreitol, 10 mM EDTA, and 20 mM Tris, pH 7.5) containing Complete protease inhibitor mixture (Roche Applied Science), and disrupted by brief sonication. The cell debris was removed by centrifugation. The proteins were separated by 7.5 or 6% SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp.). The membranes were blocked with 3% bovine serum albumin in PBS and incubated with rabbit anti-type I or VI collagen antibody (Rockland Immunochemicals, Inc.), rat anti-mouse α2(IV) collagen NC1 domain monoclonal antibody (H22) (23), or mouse anti-α-tubulin antibody (Sigma), followed by horseradish peroxidase-conjugated anti-rabbit IgG (P.A.R.I.S), anti-mouse IgG (Zymed Laboratories Inc.), or anti-rat IgG (Dako). Immunocomplexes were visualized using ECL or ECL Plus reagent (Amersham Biosciences). The protein concentrations were measured with the Bradford assay (Bio-Rad), and equal amounts of soluble protein were loaded onto the gel. The sizes of the collagen chains were determined by comparison with molecular mass standards.

Immunofluorescence Staining—For collagen staining, cells were fixed with 95% ethanol and 5% acetic acid, and for other stainings, with 4% paraformaldehyde. After blocking with 1% bovine serum albumin and 0.05% saponin in PBS, the cells were incubated with rabbit anti-type I or VI collagen antibody, rabbit anti-mouse type IV collagen antibody (Chemicon International), rabbit anti-calnexin antibody (StressGen), or mouse anti-GM130 antibody (Transduction Laboratories), followed by Alexa Fluor 488-conjugated anti-rabbit IgG or anti-mouse IgG (Molecular Probes).

Cycloheximide Treatment—LH3 knock-out and wild-type embryonic mouse fibroblasts were treated with 10 μg/ml cycloheximide for 0, 1, 2, 3, 4, or 6 h in serum-free culture medium, and cells and medium were collected after each time point. Cell lysates and trichloroacetic acid-precipitated medium proteins were used for immunoblotting. Immunofluorescence staining were performed as described above. Quantification of type IV collagen and α-tubulin at different time points was performed with ImageQuant 5.2 software (GE Healthcare). Collagen amounts were normalized with α-tubulin at each time point and calculated as a percentage of total collagen in untreated (0 h) samples.

Type VI Collagen Biosynthetic Labeling and Analysis—Biosynthetic labeling of embryonic mouse fibroblasts and newborn skin fibroblasts and immunoprecipitation of type VI collagen with rabbit anti-mouse recombinant α3(VI) collagen N1 subdomain antibody were done as described previously (24).

Histology and Immunohistochemistry—The tissues were fixed in 4% paraformaldehyde in PBS or in 10% neutral formalin overnight and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin.

For immunohistological staining, the embryos and tissues were fixed in 95% ethanol and 5% acetic acid overnight and processed for paraffin embedding. Sections were incubated with rabbit anti-type I or VI collagen and rabbit anti-mouse type IV collagen polyclonal antibodies. The primary antibody was detected with fluorescently labeled Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes).

Transmission and Immunoelectron Microscopy—Samples for transmission electron microscopy (EM) were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon 812/EMbed 812. Thin sections were cut with a Reichert Ultracut ultramicrotome. For immuno-EM, samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) with 2.5% sucrose. They were then immersed in 2.3 M sucrose and frozen in liquid nitrogen. For immunolabeling, thin cryosections were incubated with rabbit anti-type I, anti-mouse type IV, or anti-type VI collagen antibody or anti-mouse decorin antibody (LF-113) (25) and then with a protein A-gold complex. Sections were examined under a Philips CM100 transmission electron microscope. The calculations and measurements were done using the skin of three newborn LH mutant and control mice. The amounts of type IV collagen immunocomplexes were calculated from 180 10-μm^2 areas, and distances between the complexes were measured 400 times from both genotypes. Statistical analysis was performed using Student's *t* test.

RESULTS

Characterization of LH3 Knock-out Mice

The Lysyl Residues of Collagens Are Insufficiently Modified in Homozygous LH3 Knock-out Embryonic Fibroblasts—The collagens produced by LH3 knock-out embryos were character-

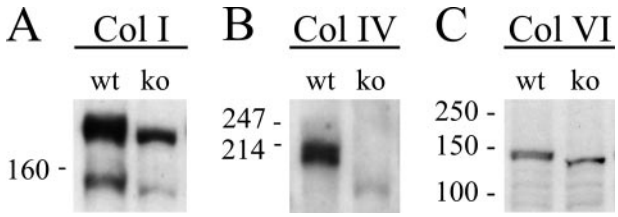

FIGURE 1. **Enhanced migration of collagens upon SDS-PAGE indicates lack of glycosylation of hydroxylysines in LH3 knock-out mice.** Shown are the results from immunoblot analysis of type I (*A*), IV (*B*), and VI (*C*) collagens (*Col*) of LH3 knock-out (*ko*) and wild-type (*wt*) embryonic mouse fibroblast cell lysates. The sizes of the collagen chains were determined by comparison with molecular markers (shown in kilodaltons). The size differences between collagens of the LH3 knock-out and control cells correspond to loss of all glycosylated hydroxylysines (see "*Characterization of LH3 Knock-out Mice*" under "Results") in the collagen chains. Equal amounts (50 μg (*A*), 86 μg (*B*), and 51 μg (*C*)) of soluble protein from knock-out and wild-type cells were loaded onto the gel.

ized using embryonic fibroblasts isolated from homozygous embryonic day 8.5 LH3 knock-out embryos. Immunoblots of type I, IV, and VI collagens showed that the collagens produced by LH3 knock-out fibroblasts migrated faster upon SDS-PAGE compared with the wild-type polypeptides (Fig. 1). The number of lost glucosylgalactosylhydroxylysyl residues was calculated using the theoretical molecular mass of 360 Da and the size difference of each collagen chain determined from the immunoblot. The size differences between the wild-type and LH3 knock-out collagens were 6.7 and 4.4 kDa for the α1(I) and α2(I) collagen chains, respectively; 14.6 kDa for the α2(IV) collagen chain; and 4.2 kDa for the α1/α2(VI) collagen chain.

The determined size differences between the wild-type and LH3 knock-out collagens correspond approximately to the calculated loss of 17, 11, 40, and 10 glucosylgalactosylhydroxylysyl residues in the LH3 knock-out α1(I), α2(I), α2(IV), and α1/α2(VI) collagen chains, respectively. The size difference between the wild-type and the LH3 knock-out type IV collagens corresponds to the previously published type IV collagen glycosylation content, whereas the size difference of type I collagen exceeds earlier observations of type I collagen glycosylation, probably because of the higher level of glycosylation of embryonic collagens (18). The amount of hydroxylysines and glycosylated hydroxylysines in embryonic mouse type I collagen chains is not known, but the α1(I) and α2(I) chains contain 24 and 21 lysines, respectively, in the *Y*-position, which can be hydroxylated and glycosylated. The size of the LH3 knock-out type VI collagen corresponds to the previously published type VI collagen lacking all the post-translational modifications of prolyl and lysyl residues (26). The immunoblot data thus suggest that the smaller size of the collagens is due to the total loss of hydroxylysine glycosylations in the LH3 knock-out cells. In addition, the results show that the LH3 knock-out fibroblasts have less type I and IV collagens than the wild-type fibroblasts; especially the amount of type IV collagen was clearly decreased in the LH3 knock-out cells. Similarly, the LH3 knock-out embryos also had a lower amount of type IV collagen than the wild-type embryos (see below).

The Secretion of Type IV Collagen Is Disrupted in LH3 Knock-out Embryos—Immuno-EM revealed abnormal type IV collagen localization in the LH3 knock-out mice. In the embryos, most of the gold particles were retained inside cells in different parts of the secretory pathway (data not shown). In the extracellular space, no aggregates and only very few individual gold particles were seen, but not in the basement membrane zone. It is also noteworthy that the total amount (both intra- and extracellular gold particles) of type IV collagen was lower in the LH3 knock-out embryos than in the wild-type embryos (data not shown). The accumulation of type IV collagen disturbed the secretion machinery of the cells, which was seen as a dilatation of the endoplasmic reticulum and a condensed Golgi structure (data not shown).

Type IV and VI Collagens Are Abnormally Distributed in LH3 Knock-out Fibroblasts—The distribution of collagens was further studied with embryonic mouse fibroblasts. Upon immunofluorescence staining, the LH3 knock-out fibroblasts showed mainly intracellular localization of type IV and VI collagens, and only a few extracellular structures were found. The data suggest defective secretion of type IV and VI collagens. In addition, the staining intensity was lower in the LH3 knock-out cells than in the wild-type cells, indicating decreased amounts of these collagen types (data not shown). The endoplasmic reticulum and Golgi staining of LH3 knock-out cells was comparable with that of controls (data not shown).

Type IV Collagen Is Abnormally Secreted from LH3 Knock-out Fibroblasts—The secretion of type IV collagen was studied in the wild-type and LH3 knock-out fibroblasts by blocking protein synthesis with cycloheximide. In the wild-type fibroblasts, the amount of type IV collagen decreased in cells and increased in the medium during treatment (Fig. 2*A*). A low amount of type IV collagen was detected in the cycloheximide-treated LH3 knock-out cells, but none at all in the medium, suggesting that most of the unglycosylated type IV collagen was degraded intracellularly. The amount of type IV collagen decreased rapidly in the LH3 knock-out cells after 1 h of cycloheximide treatment, probably because of rapid intracellular degradation. LH3 knock-out cells also showed prolonged intracellular accumulation of type IV collagen compared with wild-type cells (Fig. 2*B*). These results indicate that, because of deficient glycosylation, processing of type IV collagen is delayed after translation, which leads to its intracellular accumulation and delayed secretion. Our data also suggest that unglycosylated type IV collagen is degraded inside the cells, which also explains its decreased amount in LH3 knock-out cells and embryos.

Tetramerization and Secretion of Type VI Collagen Are Disturbed in LH3 Knock-out Fibroblasts—Tetramerization and secretion of type VI collagen were studied in embryonic mouse fibroblasts by metabolic labeling and immunoprecipitation. It has been shown previously that type VI collagen tetramers are formed inside the cells and are then secreted into the extracellular space (26, 27). Strikingly, no type VI collagen tetramers were detected in the LH3 knock-out cells or medium (Fig. 2*D*), indicating that unglycosylated type VI collagen cannot form tetramers. Because type VI collagen tetramers were not formed, the secretion of type VI collagen was also disrupted in LH3 knock-out fibroblasts. Therefore, only low amounts of α1(VI) and α2(VI) collagen chains were secreted into the medium (Fig. 2*C*).

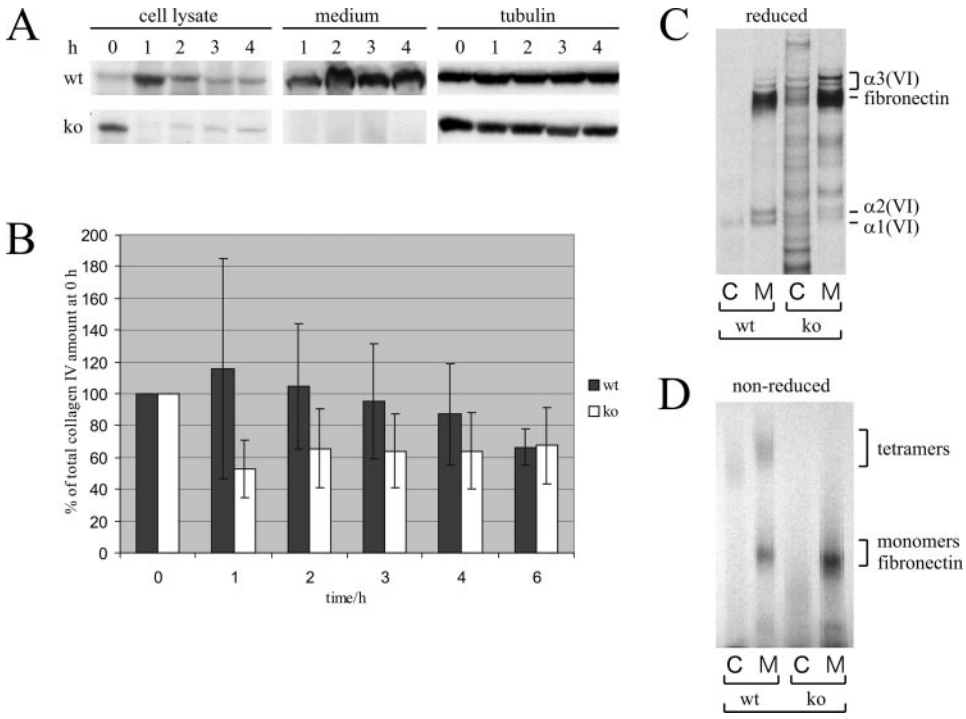

FIGURE 2. **Secretion of highly glycosylated collagen types is disrupted in LH3 knock-out fibroblasts.** *A*, cycloheximide-treated LH3 knock-out (*ko*) and wild-type (*wt*) cell lysates at different time points were immunoblotted with type IV collagen and α-tubulin. The proteins from the treated media were precipitated with trichloroacetic acid and immunoblotted with type IV collagen. *B*, the amount of type IV collagen in cycloheximide-treated LH3 knock-out and wild-type embryonic fibroblasts was compared with that in untreated cells. The amount of type IV collagen at different time points was normalized with α-tubulin and calculated as a percentage of the total collagen amount in the 0-h sample. *C* and *D*, shown are the results from electrophoretic analysis of type VI collagen produced by LH3 knock-out and wild-type embryonic mouse fibroblasts. The cells were biosynthetically labeled, and type VI collagen was immunoprecipitated from the cell (*C*) and medium (*M*) fractions and analyzed under reducing (*C*) and nonreducing (*D*) conditions. The LH3 knock-out cells secreted lower amounts of α1(VI) and α2(VI) than the control cells, and no type VI collagen tetramers were detected in LH3 knock-out cells or medium. Fibronectin in *C* and *D* was bound nonspecifically to protein A-Sepharose beads (48).

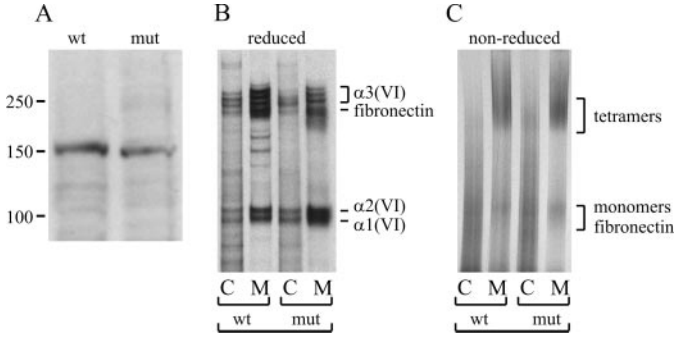

FIGURE 3. **Type VI collagen produced by LH mutant skin fibroblasts has a slightly reduced molecular mass, but is secreted as a tetramer.** *A*, immunoblot analysis of type VI collagen from LH mutant (*mut*) and wild-type (*wt*) skin fibroblast lysates. The sizes of the collagen chains were determined by comparison with molecular markers (shown in kilodaltons). The size difference indicates that the LH mutant lacks some of the modified lysyl residues (see "*Characterization of LH Mutant Mice*" under "Results"). Equal amounts (51 μg) of soluble protein from mutant and wild-type cells were loaded onto the gel. *B* and *C*, electrophoretic analysis of type VI collagen produced by LH mutant and wild-type skin fibroblasts. The cells were biosynthetically labeled, and type VI collagen was immunoprecipitated from the cell (*C*) and medium (*M*) fractions and analyzed under reducing (*B*) and nonreducing (*C*) conditions. Normal amounts of individual type VI collagen chains and collagen VI tetramers were detected in cell and medium fractions of the LH mutant cells. Fibronectin in *B* and *C* was bound nonspecifically to protein A-Sepharose beads (48).

Characterization of LH Mutant Mice

Some Lysyl Residues of Collagens Lack Modifications in LH Mutant Skin Fibroblasts—Skin fibroblasts were isolated from the newborn LH mutant and control mice to analyze the function of LH activity of LH3 in collagens. The LH mutant mice lacked the LH activity of LH3 but had normal glucosyltransferase activity and normal amounts of LH1 and LH2. The immunoblots of collagens demonstrated that highly glycosylated type IV (data not shown) and VI collagens of LH mutant skin fibroblasts migrated slightly faster than collagens from normal skin fibroblasts. The size difference between the collagens produced by mutant and control cells was best seen in type VI collagen, this difference being 1.4 kDa (Fig. 3*A*). This indicates that some hydroxylysines and subsequent glycosylations are missing despite normal glucosyltransferase activity. The difference suggests that normal levels of LH1 and LH2 do not fully compensate for the LH activity of LH3.

Type VI Collagen Tetramers Are Formed in LH Mutant Fibroblasts—The secretion and tetramerization of type VI collagen were analyzed in skin fibroblasts by metabolic labeling. The secretion of individual type VI collagen chains (Fig. 3*B*) and tetramerization of type VI collagen (Fig. 3*C*) showed no obvious abnormalities in the LH mutant compared with the control.

Type VI Collagen Is Abnormally Distributed in the Skin of LH Mutant Mice—Because the LH mutant mice show disorganized collagen fibrils and loose packing of collagen bundles in newborn skin (16), the distribution of different molecules affecting the organization of collagen fibrils was analyzed in the skin of newborn mice by immuno-EM. The distributions of type I collagen (Fig. 4, *A* and *B*) and decorin (data not shown) were similar in the LH mutant and control skin. Nevertheless, the distribution of type VI collagen was abnormal in the LH mutants. Type VI collagen was detected on the surface of the collagen fibrils in both the LH mutant (Fig. 4*D*) and control (Fig. 4*C*) skin, but in the LH mutant skin, it was less evenly distributed. The most striking defect in the LH mutant skin was the extensive aggregation of type VI collagen in the extracellular space (Fig. 4*F*). In addition, some individual gold particles or aggregates of type VI collagen were seen inside the cells (Fig. 4*F*). No aggregates or intracellular accumulation of type VI collagen was seen in the controls (Fig. 4*E*).

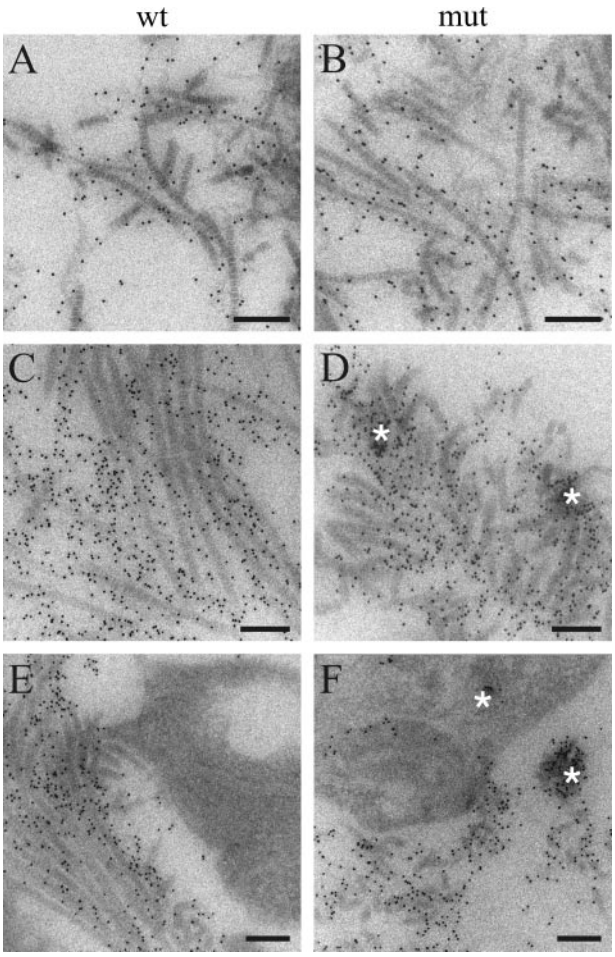

FIGURE 4. **Type VI collagen is aggregated and unevenly distributed in LH mutant skin.** Newborn skin was analyzed by immuno-EM with anti-type I and VI collagen antibodies. The distribution of type I collagen was similar in the wild-type (*wt*; *A*) and LH mutant (*mut*; *B*) mice. Type VI collagen was unevenly distributed on the surface of collagen fibrils (*) in the LH mutant mice (*D*) compared with the wild-type mice (*C*). The LH mutant skin (*F*) also showed extracellular and intracellular aggregates (*) of type VI collagen that were not detected in the wild-type skin (*E*). *Scale bars* = 0.2 μm.

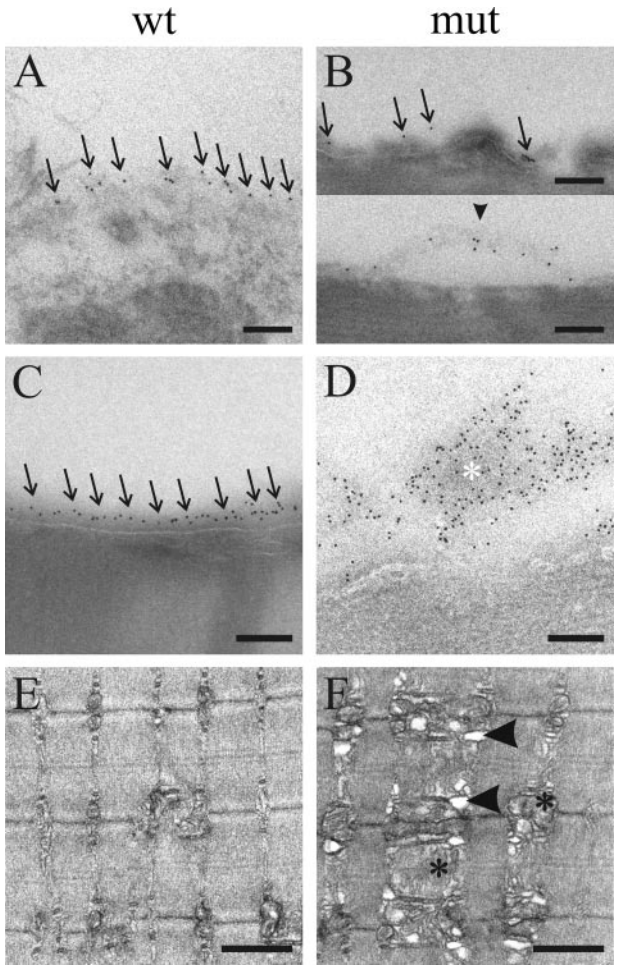

FIGURE 5. **Uneven distribution of type IV and VI collagens contributes to basement membrane detachment and abnormal ultrastructure in LH mutant skeletal muscle.** Skeletal muscle was analyzed by immuno-EM with anti-type IV (*A* and *B*) and VI (*C* and *D*) collagen antibodies and by transmission EM (*E* and *F*). Gold particles representing type IV collagen (*arrows*) were evenly distributed in the wild-type (*wt*) basement membrane (*A*), whereas the particles were unevenly scattered in the LH mutant (*mut*) basement membrane (*B*, *upper panel*). Type VI collagen was evenly distributed in the basement membrane in wild-type muscle (*C*, *arrows*), but formed aggregates in LH mutant muscle (*D*). Interestingly, immuno-EM analyses revealed detachment of the basement membrane (*arrowhead*) in LH mutant muscle (*B*, *lower panel*). The ultrastructure of skeletal muscle from LH mutant mice (*F*) showed dilatation of the sarcoplasmic reticulum (*arrowheads*) and swollen mitochondria (*) that were not detected in the wild-type mice (*E*). *Scale bars* = 0.2 μm (*A–D*) and 1 μm (*E* and *F*).

The Amount of Type IV Collagen Is Reduced in the Skin of LH Mutant Mice—Because the LH mutant mice show thinning of the epidermal lamina densa (16), the distribution of type IV collagen was also studied. Type IV collagen was detected in the epidermal basement membrane in the newborn mice, but the amount was significantly ($p < 0.0001$) lower in the LH mutant mice than in the wild-type mice. In the LH mutant mice, there were 0.72 ± 0.79 gold particles in 10 μm^2 of lamina densa, whereas in the wild-type mice, there were 1.32 ± 0.87. The decreased amount of type IV collagen was seen as an uneven distribution of type IV collagen having longer distances between individual gold particles compared with the wild-type skin. No prominent aggregation of type IV collagen was detected in the mutant or wild-type skin.

Type IV and VI Collagens Are Unevenly Distributed in Muscle and the Muscular Basement Membrane Is Detached in LH Mutant Mice—Although the immunohistochemical staining of type I, IV, and VI collagens in the skeletal muscle of adult LH mutant mice was comparable with that of wild-type mice, the distributions of these collagens were analyzed in the muscle of 18-week-old mice by immuno-EM. The data show a normal distribution of type I collagen (data not shown), but the distribution of type IV and VI collagens was altered in LH mutant muscle, as in skin. Type IV collagen was located continuously in the muscular basement membrane of the controls (Fig. 5*A*), whereas the distribution was uneven in the LH mutants (Fig. 5*B*). In LH mutant muscle, type VI collagen was unevenly distributed and heavily aggregated in the extracellular space (Fig. 5*D*) compared with wild-type muscle (Fig. 5*C*), resembling the findings in skin.

Interestingly, immuno-EM analyses revealed alterations also in the muscular basement membrane. In wild-type muscle, the basement membrane was tightly bound to the sarcolemma of the muscle fibers, whereas in LH mutant muscle, it was more distant from the muscle fibers and detached in many places (Fig. 5*B*).

The Ultrastructure of the Skeletal Muscle Is Abnormal in LH Mutant Mice—The skeletal muscle of 18-week-old mice was analyzed by hematoxylin/eosin staining. Only a few mild alterations in the structure of muscle were observed; some of the muscle fibers were less organized in LH mutant muscle compared with wild-type muscle. However, when the ultrastructure of muscles was analyzed by EM, alterations were observed in the structure of the sarcoplasmic reticulum and mitochondria of LH mutant skeletal muscle. The sarcoplasmic reticulum showed dilatations and vacuolization, and the mitochondria were swollen in the LH mutant muscles (Fig. 5*F*), and these alterations were not detected in the wild-type muscles (Fig. 5*E*).

DISCUSSION

The function of glycosylated hydroxylysines of collagenous proteins is currently not well understood. In this study, we have demonstrated by immunoblot analysis that the multifunctional LH3 enzyme catalyzes the glycosylation of hydroxylysines in several collagen types, and LH3 knock-out mice and cells thus provide a good model to study the function of glycosylated hydroxylysines. We have shown previously that loss of LH3 leads to disruption of the basement membranes and early embryonic lethality (16). In this work, we have described that the mechanism behind the disrupted basement membrane in LH3 knock-out embryos is impaired secretion and intracellular degradation of type IV collagen. A similar finding has been reported for *Caenorhabditis elegans*, where mutation of the multifunctional LH (28) leads to disrupted processing and secretion of type IV collagen (29).

Inhibition of protein synthesis revealed prolonged intracellular accumulation, delayed secretion, and intracellular degradation of type IV collagen in LH3 knock-out fibroblasts. As indicated previously, most of the unfolded collagen chains are retained in the endoplasmic reticulum and degraded intracellularly (30, 31), suggesting that the type IV collagen produced by the LH3 knock-out cells is not folded correctly. The secretion of the other highly glycosylated collagen, type VI, is also disrupted in LH3 knock-out cells because of its defective tetramerization. It can thus be concluded that the glycosylated hydroxylysines are critical for the secretion of highly glycosylated collagen types.

Our findings suggest a new role for collagen-specific hydroxylysine-linked carbohydrates in the correct folding and secretion of collagens, similar to that established for *N*-linked glycosylation of other secreted proteins (32). Although Hsp47 has been shown to be important for the secretion and folding of type IV collagen (33), the quality control of highly glycosylated collagen types has not been well understood. Decreased glycosylation of hydroxylysyl residues has also been shown to disrupt the secretion of the mannose-binding protein, a key component in the mammalian innate immune system, due to defective assembly of the collagenous triple helix (34). Recently, the multimerization and secretion of adiponectin were also shown to be dependent on the correct hydroxylation and subsequent glycosylation of lysyl residues within the collagenous domain (35, 36).

The glycosylation of hydroxylysines is also needed for the supramolecular assembly of the highly glycosylated collagen types. It has been suggested that hydroxylysine-linked disaccharides play an important role in the assembly mechanism of type IV collagen tetramers (15, 37). The importance of hydroxylysyl glycosylation to oligomerization is most clearly seen in this study because no type VI collagen tetramers were detected in our LH3 knock-out cells. Type VI collagen is unique among collagens, as it forms tetramers already inside the cells (26, 27). The post-translational modifications of type VI collagen have been so far poorly understood. It has been suggested previously that glycosylations in the helical part of type VI collagen are needed for helix-helix interactions (38). In this study, we have demonstrated for the first time that glycosylations of hydroxylysines are essential for the tetramerization of type VI collagen. Comparison of our mouse lines has demonstrated that lack of all glycosylated hydroxylysines prevents type VI collagen tetramerization and secretion, whereas lack of only some glycosylated hydroxylysines does not prevent tetramerization or secretion of type VI collagen, but causes its abnormal aggregation and distribution. It thus seems probable that most glycosylated hydroxylysines of type VI collagen are needed for its tetramerization, whereas some specific hydroxylysine glycosylations are needed for its correct distribution and probably for formation or interactions of microfibrils.

Our other mouse line, the LH mutant, has specifically inactivated LH activity but intact glycosyltransferase activities. We have shown previously that type IV and V collagen fractions extracted from the LH mutant contain 20% less hydroxylysine than the wild type (16) despite normal LH1 and LH2 levels. The substrate specificity of LH isoforms is not fully understood. Lack of some glycosylated hydroxylysines in the LH mutant despite the normal glucosyltransferase activity indicates that some of the lysyl residues are specific substrates for the LH activity of LH3. Therefore, it can be concluded that LH3 preferentially hydroxylates certain lysine residues that are further glycosylated. In addition, lack of only some glycosylated hydroxylysines suggests that LH3 can also glycosylate lysines hydroxylated by LH1 and LH2, which explains the milder defects in the LH mutant mice compared with the LH3 knock-out mice.

The LH mutant mouse line provides a good model to study the consequences of the incompletely glycosylated hydroxylysines. The analysis of LH mutant mice has demonstrated that underglycosylated type VI collagen forms aggregates and is unevenly distributed. Abnormal distribution of underglycosylated type VI collagen most likely contributes to the previously detected looser packing and disorganization of collagen fibers in LH mutant skin and lung (16) because the distribution of type I collagen was normal. Interestingly, some Ullrich congenital muscular dystrophy patients also show abnormal packing of collagen fibers (39), similar to our LH mutant. Together, the data indicate that type VI collagen is important for organization of collagen fibers. Type VI collagen has been shown to influence fibrillogenesis in several ways; it affects the arrangement of fibronectin (40), a large extracellular protein thought to be needed for fibrillogenesis (41), and it also binds to type I collagen and thus affects fibril formation (42).

The abnormal distribution of type VI collagen in the LH mutant mice also causes structural alterations in the sarcoplas-

mic reticulum and mitochondria resembling those reported previously in type VI collagen knock-out mice (43). In addition, the basement membrane of LH mutant skeletal muscle is detached and loosely bound to muscle fibers. Similarly, in some Ullrich congenital muscular dystrophy patients, collagen VI microfibrils are not linked to muscular basement membrane (44). The reduced amount of type IV collagen in the LH mutant mice may also contribute to the abnormalities of epidermal (16) and muscular basement membranes in these mice. However, our data indicate that glycosylated hydroxylysines are evidently very important for the correct distribution of type VI collagen.

The abnormalities found in LH mutant mice can also be due to abnormal collagen-collagen interactions. It has been shown that type VI collagen associates with type IV collagen in basement membranes of skeletal muscle, and defective interaction of type IV and VI collagens has been suggested to be one cause of Bethlehem myopathy, a hereditary disorder caused by mutations in the *COL6* genes (45). Because underglycosylated type VI collagen can form tetramers and is secreted, abnormal interactions between type VI collagen tetramers are a probable cause of their aggregation in the LH mutant.

The abnormalities in type VI collagen knock-out muscle have been suggested to be due to abnormal engagement of integrins (43). Similar alterations in our LH mutant propose that the lack of certain glycosylated hydroxylysines can disturb interactions between type VI collagen and integrins. Moreover, hydroxylysine-linked glycosylation of collagens has been shown previously to affect T-cell recognition of type II collagen-derived peptides (46) and the binding of type IV collagen to melanoma cell receptors (47). Together, these data suggest a role for the hydroxylysine-linked carbohydrates of collagens in cell-extracellular matrix interactions.

In this study, we have demonstrated that the lack of hydroxylysine glycosylation disturbs the secretion of type IV collagen and the tetramerization and secretion of type VI collagen. We have also shown that deficient hydroxylation and glycosylation of type IV and especially type VI collagens cause abnormal distribution of these collagen types in tissues. These results provide new insight into the role of the hydroxylysine-linked carbohydrates of collagens suggesting that they have a similar role in the correct folding and quality control of proteins as established previously for *N*-linked glycosylations. Furthermore, the results indicate that these sugars are needed for the supramolecular assembly of collagens and may also play a role in collagen-collagen interactions and in the interactions of collagens with other proteins, *e.g.* integrins. Our data expand the knowledge of collagens by demonstrating that their post-translational modifications are essential for the structure of the extracellular matrix and basement membranes. Furthermore, the striking abnormalities in our LH3-manipulated mice suggest that mutations in the gene coding for LH3 may underlie extracellular matrix disorders, especially muscular diseases.

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Secretion and Assembly of Type IV and VI Collagens Depend on Glycosylation of Hydroxylysines

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