

## Identification of *PLOD2* as Telopeptide Lysyl Hydroxylase, an Important Enzyme in Fibrosis\*

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The hallmark of fibrotic processes is an excessive accumulation of collagen. The deposited collagen shows an increase in pyridinoline cross-links, which are derived from hydroxylated lysine residues within the telopeptides. This change in cross-linking is related to irreversible accumulation of collagen in fibrotic tissues. The increase in pyridinoline cross-links is likely to be the result of increased activity of the enzyme responsible for the hydroxylation of the telopeptides (telopeptide lysyl hydroxylase, or TLH). Although the existence of TLH has been postulated, the gene encoding TLH has not been identified. By analyzing the genetic defect of Bruck syndrome, which is characterized by a pyridinoline deficiency in bone collagen, we found two missense mutations in exon 17 of *PLOD2*, thereby identifying *PLOD2* as a putative TLH gene. Subsequently, we investigated fibroblasts derived from fibrotic skin of systemic sclerosis (SSc) patients and found that *PLOD2* mRNA is highly increased indeed. Furthermore, increased pyridinoline cross-link levels were found in the matrix deposited by SSc fibroblasts, demonstrating a clear link between mRNA levels of the putative TLH gene (*PLOD2*) and the hydroxylation of lysine residues within the telopeptides. These data underscore the significance of *PLOD2* in fibrotic processes.

The biosynthesis of collagen molecules involves several intracellular post-translational modifications followed by excretion and extracellular aggregation of the collagen molecules into fibrils, which are subsequently stabilized by intermolecular cross-links (1, 2). Two related routes are responsible for the formation of these collagen cross-links, namely the allsine route, in which a lysine residue in the telopeptide is converted by lysyl oxidase into the aldehyde allsine, and the hydroxyl-

lysine route, in which a hydroxylysine residue in the telopeptide is converted into the aldehyde hydroxyallysine. Subsequently, the allsine or the hydroxyallysine reacts with a Lys or Hyl residue in the triple helix to form di-, tri-, or tetrafunctional cross-links (3–6). The mature cross-links hydroxylslylpyridinoline or lysylpyridinoline are formed via the hydroxyallysine route and occur in a variety of connective tissues such as bone, tendon, ligaments, and cartilage (7). In contrast, collagen in the skin is mainly cross-linked via the allsine route. Interestingly, in fibrotic skin (lipodermatosclerosis, keloid) and organ fibrosis (lung, liver), which is characterized by an excessive accumulation of collagen, an increase in cross-links derived from the hydroxyallysine route is found (8–15). It has been shown that the amount of hydroxyallysine-derived cross-links is related to the irreversible accumulation of collagen in fibrotic tissues, indicating that collagen containing hydroxyallysine-derived cross-links is more difficult to degrade than collagen containing allsine-derived cross-links (10–12, 14, 15). Inhibition of the formation of hydroxyallysine-derived cross-links in fibrosis is therefore likely to result in the formation of collagen that is easier to degrade, thereby preventing the unwanted collagen accumulation.

The increase in hydroxyallysine-derived cross-links in fibrosis is the result of an overhydroxylation of lysine residues within the collagen telopeptides. The enzyme catalyzing the conversion of Lys into Hyl is lysyl hydroxylase (EC 1.14.11.4) (16–18). The lysyl hydroxylase family consists of lysyl hydroxylase 1 (LH1),<sup>1</sup> LH2, and LH3 encoded by procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*), *PLOD2*, and *PLOD3*, respectively (16, 19–21). Alternative RNA splicing has been described for *PLOD2* only, resulting in the splice variants LH2a and LH2b (LH2b contains an extra exon; Ref. 22). The Ehlers-Danlos syndrome type VI is characterized by mutations in *PLOD1*. Collagen of these patients shows an absence of hydroxylated lysine residues within the triple helix in combination with normal levels of hydroxylated lysine residues within the telopeptides. Therefore, it can be concluded that LH1 catalyzes the conversion of triple helical lysine residues into hydroxylysines (23, 24). The substrate specificity of LH2

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<sup>1</sup> The abbreviations used are: LH, lysyl hydroxylase; BS, Bruck syndrome; COL1A2, collagen type 1  $\alpha 2$  chain; LOX, lysyl oxidase; *PLOD*, procollagen-lysine, 2-oxoglutarate 5-dioxygenase; SSc, systemic sclerosis; TLH, telopeptide lysyl hydroxylase.

TABLE I  
Primers for mutation analysis

Primers used for sequencing are shown in boldfaced type.

Exon	Forward primer	Reverse primer	Annealing temperature
Promoter	<b>5'-CTCCAAAGCTAAGTGCAGG-3'</b>	5'-AGACAGGGATTCCAGGGGT-3'	56 °C 30"
1	<b>5'-GTCTCTGCGTTCCTCGGAGA-3'</b>	5'-AAGGGCTGTTGGATGAATGAAC-3'	56 °C 90"
2	<b>5'-TGAGGTCCTCAATTAAGTGTAGTGA-3'</b>	5'-CTTCCTTGTGAGGATFACAGATT-3'	56 °C 90"
3	<b>5'-GTACTGTTCAAGTTGATGATGTC-3'</b>	5'-GCCACCGTGCCCAACCATAT-3'	56 °C 30"
4	5'-ATGGTTTATGTGCCTAGATTCTGA-3'	<b>5'-GGAACACCAACTCACATAATACA-3'</b>	56 °C 90"
5	<b>5'-TTCTTTCATGGTGGCTGAGCTGTA-3'</b>	5'-TGATATCCAGCCAGGTGACA-3'	56 °C 30"
6	<b>5'-GCAACTATCGCAGTTTCTACCT-3'</b>	5'-CCAAATGGACATAACAAAGGAAAG-3'	56 °C 30"
7	<b>5'-CACATACACACACAGACACACG-3'</b>	5'-AAAGGCTATCACTCTGCTGAGG-3'	64 °C 30"
8	<b>5'-TAAAGGAATATACTGCTGCAGA-3'</b>	5'-ATTCCACTTACATCTACTGCAGA-3'	56 °C 90"
9	5'-TTTCAAGTGTAGAGAAGTCCCA-3'	<b>5'-CCACTGAACCTAACCCTAATGAAT-3'</b>	56 °C 90"
10	5'-TCTAAGATTTCTAGGCTACAGGC-3'	<b>5'-GTTGGCTACTGCATACGCAAC-3'</b>	60 °C 30"
11	<b>5'-CAGAAAAGTATGCTAGAGAACCA-3'</b>	5'-GTAGAACATAACTAAGTTCCTC-3'	56 °C 90"
12	5'-CAGTTTGTGTAATGAGCTTCT-3'	<b>5'-AGGATFCCAAGTGGTCTTGGG-3'</b>	60 °C 90"
13	5'-GGGGCAGTGGTTTATCTCCTA-3'	<b>5'-CACAGTGACACACCAACTGGT-3'</b>	60 °C 90"
13A	5'-AGAATACCTGAGAGAGCGGGT-3'	<b>5'-ACGCAAAACACACAGATGACTGA-3'</b>	60 °C 90"
14	5'-CAGTTGAGTGTGCTGCTATCT-3'	<b>5'-CTGGTGTGAGACAGTATCTCAT-3'</b>	60 °C 90"
15	5'-ATAAGCATATTCAGAACCAGGCA-3'	<b>5'-CTCCACTTTCACATCTTCTGTG-3'</b>	60 °C 90"
16	5'-TCATCAATTTCTGAGGTGCACCA-3'	<b>5'-AGAAACCCGCCAACTAAT-3'</b>	56 °C 30"
17	<b>5'-AGCAGATGATATACCACATTGGA-3'</b>	5'-GTTTCATGCCAGTCATTCATCCA-3'	60 °C 30"
18 <sup>a</sup>	5'-AGCAGATGATATACCACATTGGA-3'	5'-GTTTCATGCCAGTCATTCATCCA-3'	60 °C 30"
19 <sup>b</sup>	5'-AGCAGATGATATACCACATTGGA-3'	5'-GTTTCATGCCAGTCATTCATCCA-3'	60 °C 30"

<sup>a</sup> Sequence primer for exon 18 is 5'-GTCTTTCGAGGCTATTATA-3'.

<sup>b</sup> Sequence primer for exon 19 is 5'-GCTCAAATGACATAATTTG-3'.

and LH3 has not yet been elucidated, and to date no disease has been associated with defects in LH2 or LH3.

Bruck syndrome (BS; Online Mendelian Inheritance in Man (OMIM) accession number 259450), an autosomal recessive disease, is characterized by osteoporosis, joint contractures at birth, fragile bones, and short stature (25–28). Biochemical analysis of the bone of BS patients revealed an underhydroxylation of lysine residues in the telopeptides of collagen type I, whereas hydroxylation of lysine residues in the triple helix is normal (29). Together, the Ehlers-Danlos syndrome type VI and the Bruck syndrome imply that a lysyl hydroxylase must exist that specifically hydroxylates the telopeptides and that the activity of such a telopeptide lysyl hydroxylase (TLH) is decreased in BS bone. The identity of TLH had not yet been discovered.

Here we show that the gene defect in two BS families is a mutation in *PLOD2*, showing that *PLOD2* encodes TLH. The importance of TLH in fibrotic processes is demonstrated by the highly increased expression of TLH in fibroblasts cultured from the fibrotic skin of systemic sclerosis (SSc) patients. Furthermore, higher levels of pyridinoline cross-links were found in the extracellular matrix deposited by the SSc fibroblasts, which shows the link between increased TLH expression and the shift to the hydroxyallysine cross-linking pathway.

#### EXPERIMENTAL PROCEDURES

**Patients**—In this study, three families with children diagnosed as Bruck syndrome patients were investigated. Two unrelated Kurdish families (families FH and PM), both consanguineous, were described earlier (cases 1, 2, and 3, and 7 and 8, respectively) (26); the third family (family DR) has not yet been described. This Australian family, where the parents are not consanguineous, had two affected boys deceased prenatally, one affected girl deceased at 2 years, and one surviving unaffected 9-year-old girl. All these BS patients show decreased amounts of pyridinoline cross-links in bone.<sup>2</sup>

**Genotyping**—Blood samples of the three families were collected, and genomic DNA was prepared by standard methods. Microsatellite markers, obtained from the Marshfield screening set version 6, were amplified as described earlier (29). Genotypes of the previously localized BS locus, which is 17p12 (29), and the chromosomal region of *PLOD2*, which is 3q23-q24 (30), were assigned.

**Mutation Analysis**—The exon/intron organization of *PLOD2* has not been described. *PLOD1* and *PLOD3*, encoding two other members of the lysyl hydroxylase family, both consist of 19 exons (31, 32). The exon/intron boundaries of both genes are identical. Analysis of clones RP11-758I14 and RP11-274H2 showed identical exon/intron boundaries for *PLOD2*. In addition, *PLOD2* shows an extra exon (22), designated exon 13A, which is not found in *PLOD1* or *PLOD3*. To detect DNA sequence variations in exons and exon/intron boundaries of *PLOD2*, primer pairs were designed to amplify the exons, including the exon/intron boundaries; in addition, a part of the promoter region was amplified (Table I). PCR was carried out on genomic DNA from Bruck syndrome patients, unaffected parents, and a pooled healthy population (Roche Diagnostics) using LA *Taq* polymerase (TaKaRa Bio, Inc.). PCR consisted of a 3-min interval at 95 °C, 35 cycles of 95 °C for 1 min, a specific annealing temperature as listed in Table I for 90 s or 30 s, and 68 °C for 60 s. Finally, an extension step of 10 min at 68 °C was performed. Purified PCR products served as templates for cycle sequencing using the ABI PRISM™ Big Dye termination cycle sequencing ready reaction kit (Applied Biosystems) and were analyzed on the ABI PRISM™ 310 sequencer (Applied Biosystems).

**Fibroblast Cultures**—Primary fibroblast cultures from skin biopsies of six patients with diffuse cutaneous SSc were established by routine methods. Control cultures were obtained from biopsies taken from 10 healthy controls. Cells were grown in minimal essential medium alpha (αMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, and 100 units/ml streptomycin until they reached subconfluence. Cells were detached using 0.5 ml of 0.05% trypsin/0.02% EDTA per 25 cm<sup>2</sup> and used for total RNA isolation.

**Real-time PCR Analysis**—Total RNA was isolated using the RNeasy kit (Qiagen). Subsequently, the RNA was reverse transcribed into cDNA and subjected to real-time PCR amplification. Real-time PCR amplification of cDNA sequences was performed for LH1, LH2b, LH3, the α2 chain of collagen type 1 (COL1A2), and lysyl oxidase (LOX). A real time PCR was performed for the constitutively expressed β-2-microglobulin (B2M) to standardize for differences in the total amount of cDNA. Each cDNA was amplified using specific primers and specific molecular beacons (Table II and Table III, respectively) in a total reaction volume of 25 μl containing 1× PCR buffer (Applied Biosystems), 0.4 mM of each dNTP, 3.5 mM Mg<sup>2+</sup>, 500 nM each primer, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). PCR was performed in an ABI PRISM® 7700 sequence detection system and consisted of a 5-min interval at 95 °C followed by 40 cycles of 95 °C for 30 s, 56 °C for 40 s, and 72 °C for 30 s. Data were analyzed using Sequence Detector version 1.7 software.

**PCR Amplification of LH2a and LH2b**—cDNA of human liver tissue, control, and SSc fibroblasts was subjected to LH2a and LH2b PCR amplification. LH2a (145 bp) and LH2b (208 bp) were co-amplified using specific forward (5'-TTAAAGGAAAGACACTCCGATCAGAGATGA-3') and reverse primers (5'-TAGCCTTCCAATTCATGTCTATTA-

<sup>2</sup> A. J. van der Slot, A.-M. Zuurmond, H. E. H. Pruijs, D. O. Sillence, J. DeGroot, T. W. J. Huizinga, and R. A. Bank, submitted for publication.

TABLE II  
Primers for real-time PCR

Name	Forward primer	Reverse primer
LH1	5'-CAAGCGCTCAGCTCAGTTC-3'	5'-CTTCAGCAGCCGACCTTCT-3'
LH2b	5'-TTAAAGGAAAGACACTCCGATCAGAGATGA-3'	5'-AATGTTTCCGGAGTAGGGGAGTCTTTTT-3'
LH3	5'-CTGGGCTGGGAGAGAGATG-3'	5'-TCACGTCGTAGCTATCCACAAACAT-3'
COL1A2	5'-CAAGGACAAGAAACACGCTCTGGCTAGGAGAAA-3'	5'-CAGGCGCATGAAGGCAAGTTGGGTAG-3'
LOX	5'-GTGGCCGACCCCTACTACATCC-3'	5'-AGCAGCACCCGTGTGATCATAATCTC-3'
B2M	5'-TCTTGTACTACTGAATTCACCCCACTGA-3'	5'-ATCCAAATGCGGCATCTTCAACCTC-3'

TABLE III  
Molecular beacons for real-time PCR

Abbreviations used in this table are: DABCYL, 4-(4-dimethylaminophenyl)benzoic acid; FAM, 6-carboxyfluorescein.

Name	Sequence
LH1	5'-FAM-cgtgcCTAGGGGAGGACTGGAATGTGGAGAAGGgacag-DABCYL-3'
LH2b	5'-FAM-cgtgcCGTGTATAAAGTGGATCCTGATATGGCTCTTcgacag-DABCYL-3'
LH3	5'-FAM-cgtgcgTCGAACAGTTGGTGGAGGACAGAAGGTcgacag-DABCYL-3'
LOX	5'-FAM-cgtgccATG0CGCGGGGAGGAAAAGTgacag-DABCYL-3'
COL1A2	5'-FAM-cgtgccGGCAGCCAGTTGAATATAATGTTGAAGGAggacag-DABCYL-3'
B2M	5'-HEX-cgtgcCCTGCCGTGTGAACCATGTGACTTTGgacag-DABCYL-3'

GAAATGTA-3') in a total reaction volume of 25  $\mu$ l containing 1 $\times$  PCR buffer (Applied Biosystems), 0.4 mM each dNTP, 3.5 mM Mg<sup>2+</sup>, 500 nM each primer, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). PCR was performed in a 9600 thermal cycler (PerkinElmer Life Sciences) and consisted of a 5-min interval at 95 °C followed by 35 cycles of 95 °C for 30 s, 56 °C for 40 s, and 72 °C for 30 s. Aliquots of 15  $\mu$ l of each amplified sample were subjected to electrophoresis in a 2% agarose gel containing ethidium bromide.

**Cross-link Analysis**—Measurements were performed on extracellular matrix obtained from fibroblasts cultured for 5 weeks in the presence of ascorbic acid (50  $\mu$ g/ml). Pyridinoline cross-links were measured in acid hydrolysates of unreduced samples by reversed-phase high-performance liquid chromatography (33, 34).

**Statistical Analysis**—Statistical analysis was performed using SPSS 11.0 software. Cross-link analysis and real time PCR data are presented as mean  $\pm$  standard deviation. Patients and controls were compared by Student's *t* test (two-tailed).

**GenBank™ Accession Numbers**—Accession numbers are as follows: chromosome 3 clone RP11-758114, AC053539; chromosome 3 clone RP11-274H2, AC018369; *PLOD1*, NM\_000302; *PLOD2*, U84573; *PLOD3*, XM\_051244; *COL1A2*, XM\_004658; *B2M*, XM\_007650; and *LOX*, NM\_002317.

## RESULTS

**Linkage Analysis of BS Families**—To decipher the gene encoding TLH, the gene defect in BS patients was examined. In a previous paper we mapped the *BS* locus on an 18-centimorgan region on chromosome 17p12 (29). At that time, only a single family was available for genotyping because of the extreme rarity of the disease. To refine the candidate region, two other diagnosed BS families (family DR and family PM) were now screened for linkage to chromosome 17p12. Surprisingly, genotype analysis excluded the linkage of both families to chromosome 17p12. This implies that, in addition to a *BS* locus on chromosome 17p12, a *BS* locus on another chromosomal region exists.

We reasoned that the candidate region for the second *BS* locus is chromosome 3q23-q24, as *PLOD2* encoding LH2 is located in that region (30). This assumption was based on the observation of a parallel increase of LH2 expression and hydroxylysine-derived cross-links in osteogenic progenitor cells during the course of *in vitro* differentiation (35), which suggests that LH2 catalyzes the conversion of the telopeptide lysine residue into hydroxylysine. Indeed, haplotype analysis of family DR and family PM revealed that both families confirm the candidate status of chromosome 3q23 (Fig. 1, *a* and *b*).

**Mutation Analysis of BS Families**—To verify that the *BS* phenotype in the patients in family DR and family PM is indeed due to mutations in *PLOD2*, a mutation analysis was performed. In both patients of family DR a homozygous mis-

sense mutation in exon 17 of C $\rightarrow$ T was found, resulting in a Thr  $\rightarrow$  Ile substitution at position 608 and 629 (LH2a and LH2b, respectively). Both parents and the healthy sister were carriers of the mutation (Fig. 2*a*). In the same exon a homozygous missense mutation of G $\rightarrow$ T was observed in both patients of family PM, resulting in a Gly  $\rightarrow$  Val substitution at positions 601 and 622 (LH2a and LH2b, respectively). The parents were heterozygous for the mutation (Fig. 2*b*). Neither mutation was detected in a pool of 250 chromosomes of healthy individuals, thereby excluding the possibility that these mutations represent polymorphisms. Mutation analysis of patients from the BS family (family FH) showing linkage to chromosome 17p12 (29) did not reveal any mutations in the exons, exon/intron boundaries, and the promoter region of *PLOD2*.

**TLH Expression in SSc Fibroblasts**—Because BS indicates that the specific substrate of LH2 is the lysine residue within the collagen telopeptides, we were interested in finding out whether the expression of LH2 is increased in cells present in fibrotic lesions, as the collagen deposited in fibrotic lesions shows an increased hydroxylation of the telopeptides. Real-time PCR analysis of mRNA extracted from SSc skin fibroblasts revealed that the expression of LH2b mRNA was highly increased in these fibroblasts compared with controls (Fig. 3*a*; controls 0.02  $\pm$  0.01; SSc patients 0.15  $\pm$  0.06; *p* = 0.003). Interestingly, LH1 (Fig. 3*b*; controls 0.61  $\pm$  0.23; SSc patients 1.08  $\pm$  0.82) and LH3 (Fig. 3*c*; controls 0.05  $\pm$  0.02; SSc patients 0.09  $\pm$  0.07) expression levels were unchanged. Expression of LH2a mRNA was undetectable both in controls and SSc patients (Fig. 3*d*). In agreement with previous data (36, 37), elevated levels of COL1A2 mRNA were found in SSc fibroblasts (Fig. 3*e*; controls 3.98  $\pm$  2.01; SSc patients 16.53  $\pm$  7.17; *p* = 0.007). No changes in the expression of LOX mRNA (Fig. 3*f*; controls 0.43  $\pm$  0.31; SSc patients 0.49  $\pm$  0.24) were observed. Finally, elevated levels of LH2b mRNA expression relative to COL1A2 mRNA expression were found in SSc fibroblasts (controls 0.0057  $\pm$  0.0034; SSc patients 0.0098  $\pm$  0.0021; *p* = 0.009), whereas LH1 (controls 0.18  $\pm$  0.088; SSc patients 0.06  $\pm$  0.04; *p* = 0.002), LH3 (controls 0.015  $\pm$  0.007; SSc patients 0.005  $\pm$  0.004; *p* = 0.002), and LOX (controls 0.12  $\pm$  0.08; SSc patients 0.03  $\pm$  0.005; *p* = 0.004) mRNA expressions relative to COL1A2 expression were decreased.

Analysis of the collagen, deposited by fibroblasts cultured from fibrotic skin of SSc patients, showed an increase in hydroxylysine-derived cross-links, indicating that the hydroxylation of the collagen telopeptides is also increased in the fibrotic skin of SSc patients (Fig. 4; controls 0.041  $\pm$  0.017; SSc

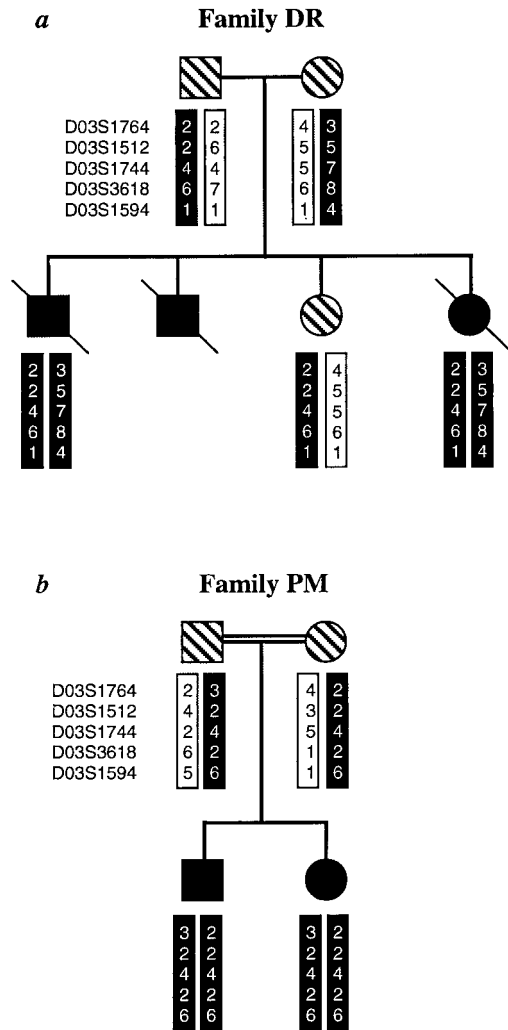


FIG. 1. Haplotype analysis in two BS families, family DR (a) and family PM (b), using five microsatellite markers on 3q23. Black symbols denote affected individuals, and striped symbols denote carriers of the disease. The haplotypes co-segregating with the disease are indicated by a black box. Consanguinity is indicated with a double black line.

patients  $0.15 \pm 0.049$ ;  $p = 0.016$ ). This is in line with previous *in vivo* data (38).

#### DISCUSSION

Fibrosis is characterized by an excessive accumulation of collagen. Furthermore, a change in the collagen cross-link chemistry is found in fibrotic lesions. Therapies to reduce collagen accumulation in fibrosis are mostly focused on the reduction of collagen synthesis and the induction of proteinase activity. A drawback of these approaches is that not only the pathological but also the physiological collagen production and degradation in the surrounding tissue are affected. Recently, it has been postulated that the change in cross-linking, giving a decrease in the proportion of allysine-derived cross-links in favor of hydroxyallysine-derived cross-links, is a unique process in fibrosis (8–15). The amount of hydroxyallysine-derived cross-links (such as pyridinolines) is related to the irreversible accumulation of collagen in fibrotic tissues (10–12, 14, 15). This suggests that collagen containing hydroxyallysine-derived cross-links is more difficult to degrade and therefore contributes significantly to the accumulation of collagen. The increased amount of pyridinolines in fibrosis is the consequence of an overhydroxylation of the lysine residues within the telo-

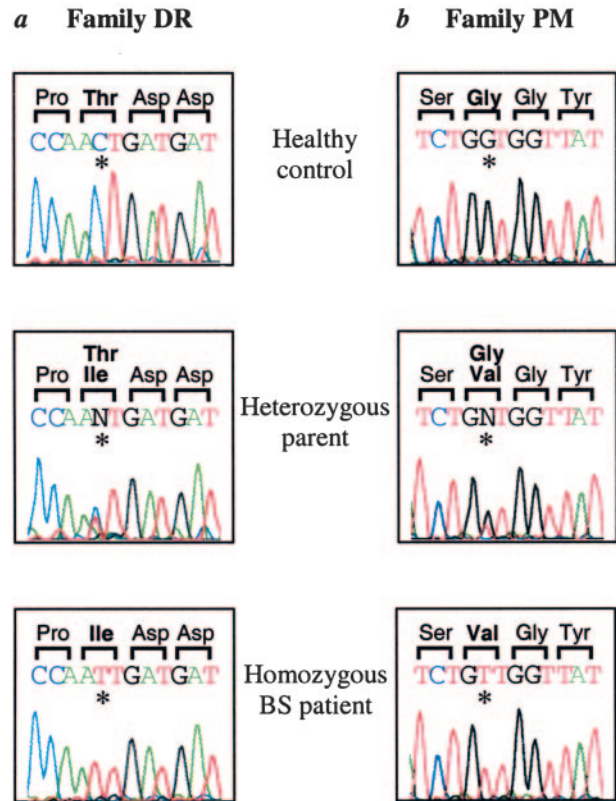


FIG. 2. Missense mutations in *PLOD2* lead to BS phenotype. a, 1823C→T/1886C→T mutation (in the cDNA sequence of LH2a and LH2b, respectively) in family DR. A sequence chromatogram of exon 17 from a healthy control is shown aligned with those of a heterozygous healthy parent and a homozygous BS patient. This mutation leads to a Thr → Ile substitution at positions 608(LH2a) and 629(LH2b). b, 1802G → T/1865G→T mutation in family PM. The sequence chromatograms of exon 17 show the sequence of a healthy control, a heterozygous healthy parent, and a homozygous BS patient. This mutation results in a Gly → Val substitution at position 601 (LH2a) and 622 (LH2b).

peptides. Although the existence of a TLH has been postulated (24, 29), the gene encoding TLH has, to date, not been identified.

Biochemical analysis of the bone of BS patients revealed that the BS phenotype is due to the decreased activity of TLH (29). Three lysyl hydroxylases are known, *i.e.* LH1, LH2, and LH3, encoded by *PLOD1*, *PLOD2*, and *PLOD3*, respectively. LH1 is a helical lysyl hydroxylase (23, 24), whereas the substrate specificity of LH2 and LH3 is unknown. Here we show, by means of mutation analysis, two different missense mutations in exon 17 of *PLOD2* in BS patients, indicating that LH2 is the putative TLH. These mutations are situated in an amino acid sequence showing high homology between the different lysyl hydroxylases, LH1, LH2, and LH3, and between the lysyl hydroxylases of species ranging from *Homo sapiens* to *Caenorhabditis elegans* (Fig. 5). Such a strict conservation implies that this region is important for the function of the lysyl hydroxylases in general and that of LH2 in particular.

Mutation analysis of patients from a BS family showing linkage to chromosome 17p12 (29) did not reveal any mutations in the exons, exon/intron-boundaries, or the promoter region of *PLOD2*. This suggests that these patients must have another defect. Patients linked to chromosome 17 should be called Bruck syndrome type I, whereas patients showing mutations in *PLOD2* belong to the Bruck syndrome type II. This subdivision is solely based on genetic data, as we are not aware of phenotypic differences.

BS provides, for the first time, insight into the substrate

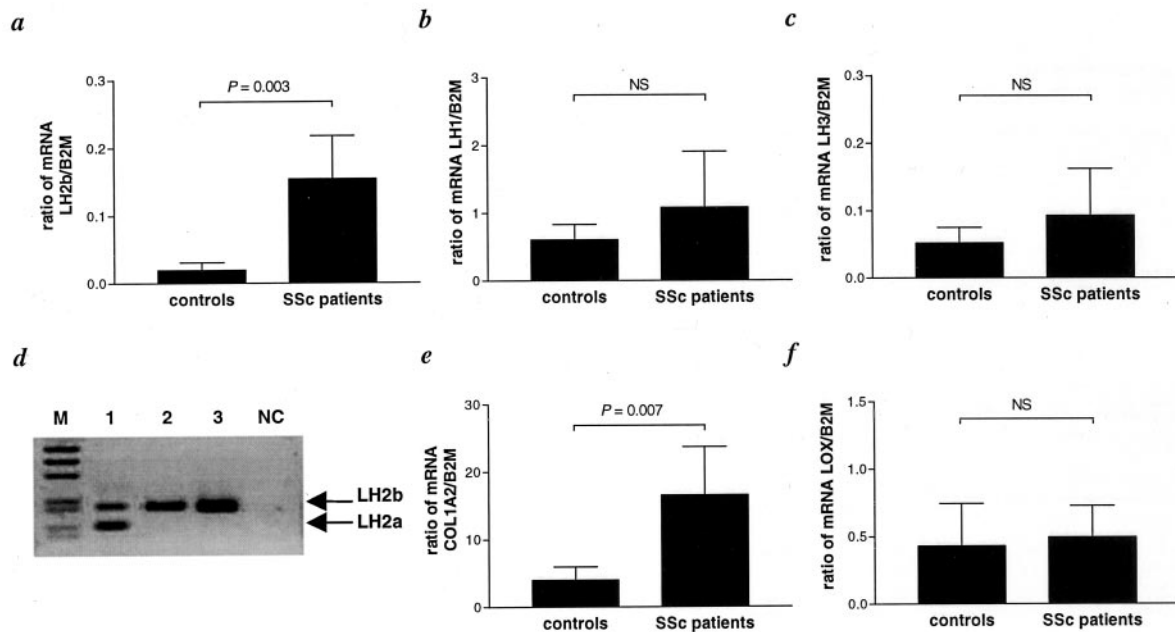


FIG. 3. Increased LH2b and COL1A2 mRNA expression in SSc fibroblasts. Shown is mRNA expression of LH2b/B2M (a), LH1/B2M (b), LH3/B2M (c), COL1A2/B2M (e), and LOX/B2M (f) in control ( $n = 10$ ) and SSc fibroblasts ( $n = 6$ ). Expression of LH2a and LH2b in liver (1), control fibroblasts (2), and SSc fibroblasts (3) is shown in panel d. NC, negative control; M, molecular weight marker.

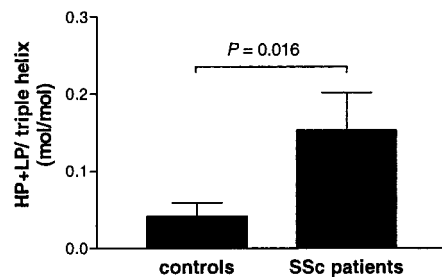


FIG. 4. The pyridinoline cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) in the extracellular matrix deposited by SSc fibroblasts ( $n = 4$ ) are increased as compared with control fibroblasts ( $n = 7$ ).

specificity of LH2; it is a telopeptide lysyl hydroxylase. As the increased amount of pyridinolines in fibrosis is likely to be the results of increased TLH activity, we investigated the expression of LH2 in fibrotic tissues. Analysis of LH1, LH2a, LH2b, and LH3 mRNA expression in fibrotic skin of SSc patients revealed that, of the collagen-modifying enzymes, only the mRNA level of LH2b relative to COL1A2 was increased. LH2a was undetectable in both control and SSc fibroblasts, indicating that this splice variant is absent in skin fibroblasts and therefore plays no role in fibrosis. Furthermore, we found normal mRNA levels of LOX, which is the enzyme catalyzing the formation of allysine and hydroxyallysine, a step necessary for the formation of cross-links. The increase in pyridinoline cross-links as found in our fibroblast cultures of SSc patients is therefore not due to increased aldehyde formation. Together, these results imply that only LH2b can be responsible for the overhydroxylation of collagen telopeptide lysine residues in SSc skin, leading to the increased formation of pyridinolines. It is therefore reasonable to assume that LH2b plays an important role in the irreversible accumulation of collagen in fibrosis.

The type of cross-links provides a mechanism for regulating the rate of collagen catabolism (10–12, 14, 15). The inhibition of the formation of hydroxyallysine-derived cross-links by specifically inhibiting TLH in order to favor the formation of allysine-derived cross-links is likely to make the collagen more

<i>Homo sapiens</i> LH1	R I Q G G Y E N V P T I D I H M
<i>Homo sapiens</i> LH2	R I S <b>G</b> G Y E N V P <b>T</b> D D I H M
<i>Homo sapiens</i> LH3	R L A G G Y E N V P T V D I H M
<i>Bos taurus</i> LH1	R I Q G G Y E N V P T I D I H M
<i>Rattus norvegicus</i> LH1	R I Q G G Y E N V P T I D I H M
<i>Mus musculus</i> LH1	R I Q G G Y E N V P T I D I H M
<i>Mus musculus</i> LH2	R I S G G Y E N V P T I D I H M
<i>Mus musculus</i> LH3	R L A G G Y E N V P T V D I H M
<i>Gallus gallus</i> LH1	R I Q G G Y E N V P T I D I H M
<i>Drosophila melanogaster</i> LH	R L E G G Y E A V P T R D I H M
<i>Caenorhabditis elegans</i> LH	R L A G G Y E N V P T R D I H M

FIG. 5. Alignment of the sequence region of human LH2, where both mutations (black boxes) are located, shows strong homology (gray boxes) with the sequence of the different human LH isoenzymes and the LH isoenzymes of different species.

susceptible to proteolytic degradation, leading to a decrease in collagen accumulation. An additional advantage of this approach is that the collagen synthesis needed for normal remodeling of the tissue is not compromised, as TLH up-regulation is specifically seen in fibrosis. Inhibition of LH2 is therefore an attractive way to interfere with fibrotic processes, making LH2 a new and promising target for anti-fibrotic therapies.

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## Identification of *PLOD2* as Telopeptide Lysyl Hydroxylase, an Important Enzyme in Fibrosis

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