Lysyl Hydroxylase 3 Is a Multifunctional Protein Possessing Collagen Glucosyltransferase Activity*

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Received for publication, July 13, 2000
Published, JBC Papers in Press, August 8, 2000, DOI 10.1074/jbc.M006203200

Collagens are extracellular proteins found essentially in all tissues. They play a crucial role in maintenance of the structural integrity of tissues and in regulation of cellular behavior. The collagens, like other extracellular proteins, bind to growth factors and other regulatory components of cells and modulate cellular metabolism. To date, 19 genetically distinct collagen types have been identified (1–3). The collagen molecule is composed of three polypeptide chains, which coil around each other into a triple helical structure. Some of the collagen types, such as type I, II, and III collagens, have a rod-like structure without any interruptions in the helical region, whereas in the other types the triple-helical regions are interrupted with multiple short nonhelical sequences. Collagen molecules are aggregated in tissues into supramolecular structures such as fibrils, beaded filaments, or net-like or other kinds of structures depending on the type of collagen (3).

The biosynthesis of collagen involves several post-translational modifications, which include hydroxylation of lysyl residues, galactosylation of hydroxylysyl residues, and glucosylation of galactosylhydroxylysyl residues. These reactions occur in the endoplasmic reticulum before triple helix formation. Hydroxylysine occurs in the Y position of the repeating Xaa-Yaa-Gly triplet within the helical region of collagen molecules and also in the sequence of the nonhelical telopeptide regions of some collagen types when glycine is replaced either by serine or alanine (2, 4). The hydroxy groups of hydroxylysyl residues provide attachment sites for glycosyl residues, either the monosaccharide galactose or the disaccharide glucosylgalactose.

The hydroxy groups also play a crucial role in the formation of inter- and intramolecular collagen cross-links. The biological role of the hydroxylsyyl-linked carbohydrates, which are collagen-specific structures, is not clear. These carbohydrates point outward from the collagen helix and, thus, are located at the outside of the protein when the protein with PBS is incubated for 2 h at room temperature. Some of the carbohydrates are linked by glycosamino-glycans, and between collagen molecules and other extracellular matrix components. The number of glycosylated hydroxylysyl residues as well as hydroxylated lysyl residues is variable, both between collagen types and within the same collagen in different tissues and at different ages (2, 4).

Lysyl hydroxylase (EC 1.14.11.4) and glucosyltransferase (EC 2.4.1.66) are enzymes involved in post-translational modifications during collagen biosynthesis. We reveal in this paper that the protein produced by the cDNA for human lysyl hydroxylase isoform 3 (LH3) has both lysyl hydroxylase and glucosyltransferase (GGT) activities. The other known lysyl hydroxylase isoforms, LH1, LH2a, and LH2b, have no GGT activity. Furthermore, antibodies recognizing the amino acid sequence of human LH3 and those against a highly purified chicken GGT partially inhibited the GGT activity. Similarly, a partial inhibition was observed when these antibodies were tested against GGT extracted from human skin fibroblasts. In vitro mutagenesis experiments demonstrate that the amino acids involved in the GGT active site differ from those required for LH3 activity.

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Lysyl hydroxylase (EC 1.14.11.4) catalyzes the hydroxylation of lysyl residues in collagens in a reaction that requires Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate (4). Three enzymes (LH1, LH2, LH3) with LH1 activity have been characterized in human (5–8) and mouse tissues (9). The LH2 isoform is present in two alternatively spliced forms, LH2a and LH2b (10, 11). Galactosylhydroxylysyl glucosyltransferase (GGT, EC 2.4.1.66) adds glucose to some galactosyl hydroxylysyl residues in collagen in a reaction that requires Mn^{2+} and UDP-glucose (12). GGT has been purified from chicken embryos (13), and its catalytic and molecular properties have been characterized (12, 14). However, the enzyme has not yet been cloned. We report here a multifunction of LH3. The protein produced by the LH3 cDNA has both LH and GGT activities.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Sf9 (Spodoptera frugiperda) insect cells were grown in SF900 II serum-free medium (Life Technologies, Inc.). Expression of recombinant protein was carried out by the baculovirus transfer vector (15) in the BAC-TO-BAC™ expression system (Life Technologies, Inc.).

COS-7 cells were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. 8 × 10^5 cells/100-mm dish or 1 × 10^6 cells/35-mm dish were plated 1 day before transfection. Cells were transfected by plasmid DNA using FUGENE6 (Roche Molecular Biochemicals). After incubation for 0–48 h, the cells were harvested and washed twice with PBS.

Expression of LH3 cDNA in Insect and Mammalian Cells—Commercially available baculovirus transfer vector pFastBac1 in the BAC-TO-BAC™ expression system was modified to contain the human LH1 signal peptide (5), a His tag, and a BamHI restriction site for insertion of the desired cDNA, nucleotides 289–2455 of LH3 cDNA (7). The construct was confirmed by sequencing. The recombinant protein contains the His tag at the amino terminus after signal peptide cleavage. Insect cells were harvested 0–72 h after infection and homogenized according to the protocol described earlier (6).

Expression of LH3 cDNA was also carried out in a mammalian expression vector as LH3 and as a LH3-GFP fusion protein. The human

* This work was supported by grants from the Research Council for Natural Sciences within the Academy of Finland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: LH, lysyl hydroxylase; GGT, galactosylhydroxylysyl glucosyltransferase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis.
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LH3-coding sequence covering the nucleotides 214 to 2447 (7) was subcloned into BamHI and XhoI sites of a pCDNA3 vector (Invitrogen) and expressed in COS-7 cells. The coding sequence was also subcloned into the EcoRI site of the pEGFP-N1 vector (CLONTECH), where a GFP tag forms the amino-terminus of the fusion protein. The intermediate vector (TGA of LH3 was mutated to TGG (Glu) by replacing A\textsuperscript{2443} to G\textsuperscript{2443} using the QuickChange site-directed mutagenesis kit (Stratagene). To maintain the correct reading frame for GFP, T\textsuperscript{2447} was simultaneously deleted from the sequence. The transfected COS-7 cells were sonicated in a solution containing 0.1% Triton X-100, 20 mM NaCl, and 20 mM Tris-HCl, pH 7.5, for 10 s in ice, and centrifuged (14,000 rpm, 30 min), and the supernatant was used for activity measurements.

Expression of LH1 and LH2 cDNA in Insect Cells—The human LH1 signal peptide and a His\textsubscript{8} tag were inserted to CDNAs constructs of LH1 and LH2. The baculovirus transfer vector pFastBac1 was modified so that human LH1 signal sequence followed by nine nucleotides of the amino-terminal end was ligated to the His\textsubscript{8} tag followed by nucleotides for BamHI restriction site. The human cDNA sequence (LH1 or LH2) starting from the likely amino-terminal end of the molecule was ligated to the BamHI site of the construct. Two expression constructs were generated for LH2, LH2a and LH2b, both of them covered the nucleotides from 76 to 2267 (6). The LH2b construct also contained the alternatively spliced exon sequence (11) between nucleotides 1500 and 1501. The constructs were confirmed by sequencing. Insect cells were grown as described above.

Purification of His-tagged Proteins by Nickel-NTA-agarose—His-tagged recombinant proteins were purified by Ni-NTA-agarose (Qiagen) using the batch purification protocol as described by the manufacturer. The agarose was equilibrated with 20 mM Tris-HCl, pH 7.8, 0.3 M NaCl, 5% glycerol, and 10 mM imidazole. Ni-NTA-agarose was mixed into the cell homogenate with additions of 5% glycerol, 0.3 M NaCl, and 10 mM imidazole, and the slurry was incubated for 45 min at 4 °C on a rocking platform. The matrix was then washed in 20 mM Tris-HCl, pH 7.8, 0.3 M NaCl, 5% glycerol, and 20 mM imidazole. Elution (incubation of the matrix for 10 min in an elution buffer) was carried out in three steps; the elution buffers contained 100, 200, and 300 mM imidazole.

Microscopical Studies—To follow expression of the LH3-GFP fusion protein in COS-7 cells, the cells transfected with the pEGFP-N1-LH3 plasmid DNA were grown on coverslips. Forty-eight hours after transfection, the cells were washed twice with PBS and briefly rinsed in distilled water. The coverslips were then mounted on slides using Immuno-mount (Shandon). The expression levels of the LH3-GFP protein were visualized directly by fluorescence microscopy (Nikon).

The transfected cells were also stained with antibody against protein disulfide isomerase (16). The cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 min, blocked for 1 h in 0.05% saponin, 0.1% bovine serum albumin in PBS buffer pH 7.4 (IF buffer), followed by a 1-h incubation at room temperature with a monoclonal antibody (1:100) against protein disulfide isomerase (DAKO). Coverslips were washed three times with IF buffer and incubated for a further 1 h with an anti-mouse IgG tetramethylrhodamine B isothiocyanate (1:100) (DAKO). After three washes with PBS and one brief rinse in distilled water, the coverslips were mounted on slides using Immuno-mount, and the staining was observed by fluorescence microscopy.

Immunoprecipitation—Cells transfected with the LH3 construct or vector alone were lysed with 5 mM EDTA, 0.5% Triton X-100 in PBS for 30 min at 4 °C. The cell extracts were pretreated with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) to remove nonspecific binding and then immunoprecipitated with monoclonal anti-GFP antibody (CLONTECH) or monoclonal anti-polyhistidine antibody (Sigma) together with protein A-Sepharose CL-4B beads. The proteins bound to beads were separated by SDS-PAGE and detected by immunoblotting or silver staining.

In Vitro Translation—For in vitro translation, the coding sequence (amino acids 33–738) of LH3 cDNA was cloned in-frame into the pCITE 4a vector (Novagen) at the EcoRI/XhoI site under the T7 promoter. This vector provides the transcribed mRNA with a cap independent translation enhancer at the 5’ end and a poly(A) tail. The plasmid was subcloned into Escherichia coli XL1-blue strain and purified with a Mini Plasmid kit (Qiagen). In vitro translation was performed with the single tube protein system 3 kit, STP3 (Novagen) according to the manufacturer’s protocol. Reaction volumes were scaled down. Briefly, for transcription, 250 ng of plasmid were mixed with 3.2 µl of transcription mix in a total volume of 4 µl and incubated at 30 °C for 15 min. For translation, 1.6 µl of [35S]methionine (10 µCi/ml, Amersham Pharmacia Biotech) or 0.8 µl of cold methionine in the kit and 12 µl of translation mix were added to the transcription reaction and incubated at 30 °C for 1 h. The product was analyzed by SDS-PAGE and autoradiography, and GFP activity was measured directly in the reaction mixture containing unlabeled methionine.

Activity Measurements—LH activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate (17) with the synthetic peptide IKGIKGIG used as a substrate. GFP activity was assayed as described elsewhere (18). The method is based on the transfer of radioactively (tritium)-labeled glucose from UDP-glucose to galactosylhydroxylysyl residues in a calf skin gelatin substrate and on the specific detection of the product of the enzyme reaction after alkaline hydrolysis.

RESULTS

Collagen Glucosyltransferase Activity in Cells Transfected with LH3 cDNA—We have prepared a cDNA construct of LH3 for expression of an LH3 protein with a His tag, which remains at the amino-terminal end of the protein after signal peptide cleavage. Sf9 cells were infected with this construct, and LH and GGT activity were measured in the cells at different time points (Fig. 1A). As seen in the figure, the enzyme activities began to increase about 30 h after transfection. At this same time we could begin to detect GGT activity in the culture medium. We were unable to measure LH activity, however, due to the presence of inhibitory substances in the medium (21). The rapid increase of GGT activity in the medium, approximately 50 h after transfection, seen in Fig. 1A, was probably due to cell lysis due to the infection.

We tested the transfections also in mammalian cells, where native LH3 cDNA was used in the transfections (Fig. 1B). A similar time course for GGT activity was obtained in these cells. When the transfected COS-7 cells were studied at different time points by Northern hybridization using LH3 cDNA as a probe, the data indicated a clear increase in LH3 mRNA 20 h after transfection (not shown).

The results presented in Table I indicate the activities measured in one experiment. There is a large increase in GGT activity in the LH3 cDNA-transfected Sf9 and COS-7 cells. The increase is higher in the Sf9 cells, varying from about 120 to 2500 times the background activity in different transfections. Nontransfected COS-7 cells possess high GGT activity, but after transfection, the activity increased to the background level about 4–5-fold. Expression of LH3 in both systems resulted in secretion of enzyme into the medium (Table 1). In Sf9 cells, about 25 to 55% of the activity was secreted, whereas in COS-7 cells the corresponding value was about 80%.

Intracellular Distribution of Protein Produced by LH3 cDNA—We prepared a construct for the LH3-GFP fusion protein in which GFP, a fluorescent protein, was inserted into the carboxyl-terminal end of LH3. The cells transfected with the construct produced a fusion protein, which can be visualized by fluorescence microscopy. As seen in Fig. 2A, the fusion protein was located mainly in the endoplasmic reticulum. In some cells, 

\[ T. \text{Hillukkala and M. Mäkiniemi, unpublished information.} \]
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A cell-free system can be used to study the expression of a supplemented DNA in a condition where no other DNA is present. The presence of a radioactive amino acid allows detection of even low amounts of the protein expressed in the system. We have used the cell-free system to rule out the possibility that a DNA sequence other than the LH3 cDNA is responsible for the production of GGT activity. As seen in Table II, both antibodies partially inhibited the catalytic activity of the GGT produced by the His-tagged LH3 expressed in the cell-free system. We used antibodies against a synthetic peptide corresponding to amino acids 283 to 297 of the human LH3 protein. As seen in Table II, both antibodies partially inhibited the GGT activity. However, no inhibition by anti-His tag antibodies was detected. Antibodies against human Dpb11 protein were used as controls, and these antibodies had no effect on GGT activity.

The antibodies were also tested with GGT prepared from human skin fibroblasts. LH3 and GGT antibodies partially inhibited GGT activity from human skin fibroblasts (Table II), whereas no inhibition was obtained with anti-His antibodies or control antibodies.
The Effect of Mutations in LH3 on the GGT Activity of the Protein—To determine what regions of the molecule or which amino acids of LH3 are required for GGT activity, we generated a frame shift in LH3 after leucine at position 231 by a one-nucleotide deletion. This modification of the construct produced a protein shown in Fig. 5A, where only amino acids up to 231 were similar to the LH3 sequence. This protein had neither LH nor GGT activity (Table III). Removal of 217 amino acids from the carboxyl terminus of LH3 totally eliminated LH activity, whereas the protein still retained about one-fifth of the GGT activity. Mutation of aspartate at position 669 to alanine decreased LH functionality dramatically, whereas it had no effect on the GGT activity. One nucleotide deletion after the codon for His-668 in the LH3 cDNA sequence caused a shift in the reading frame and generated a translational stop codon after 30 amino acids. In this protein, the last 70 carboxyl-terminal amino acids of LH3 are missing from the molecule. The protein had no LH activity but still retained about half of the original GGT activity. Fig. 5B shows that the mutations did not cause any significant effects on the levels of protein expression.

Expression of LH1 and LH2 in Insect Cells Does Not Generate GGT Activity—The two other lysyl hydroxylase isoforms LH1 and LH2, the latter one in two alternatively spliced forms (LH2a and LH2b), were expressed in Sf9 cells under conditions identical to those used for LH3. GGT activity was measured in cell lysates 72 h after infection (Fig. 6). The production of recombinant proteins was followed by LH activity measurements and SDS-PAGE combined with anti-His tag antibody staining. The production of recombinant proteins was at the same level (not shown). Although LH activity was present in the cell lysates, none of these recombinant proteins had detectable GGT activity.

**DISCUSSION**

We have shown in baculovirus and mammalian expression systems that the expression of LH3 cDNA produced both LH activity and GGT activity. The appearance of the activities in the cells occurs simultaneously, without any lag between the activities, which might suggest that expression of LH caused the simultaneous induction of transcription of the GGT gene.
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**TABLE II**

| Antibody   | Sf9/LH3 % | p value | FB % | p value |
|------------|-----------|---------|------|---------|
| Nonimmune serum  | 100 ± 7    | 100 ± 24 |
| LH3        | 84 ± 13    | 0.043   | 67 ± 22 | 0.046 |
| GGT        | 84 ± 12    | 0.029   | 70 ± 3 | 0.043 |
| His tag    | 100 ± 6 NS | 92 ± 23 NS |
| Dpb11      | 101 ± 22 NS | 108 ± 27 NS |

**FIG. 6.** GGT activity in different lysyl hydroxylase isoforms. All LH isoforms as His-tagged versions were produced in insect cells (see “Experimental Procedures”). GGT activity (given as disintegrations/min (DPM) above the column) was measured in cell lysate 72 h after the transfections. Sf9 cell lysate was used as a control.

**TABLE III**

| LH3 activity | GGT activity |
|--------------|--------------|
| dpm/plate    | %            | dpm/plate | % |
| LH3 construct, nonmutated | 6,000 | 100 | 760,000 | 100 |
| Mutation 1   | <300 <5      | 650 0.1  |
| Mutation 2   | <300 <5      | 180,000 | 23 |
| Mutation 3   | <300 <5      | 970,000 127 |
| Mutation 4   | <300 <5      | 360,000 | 47 |

examination revealed that in some cells the protein was retained in the endoplasmic reticulum, in agreement with the data obtained with LH1 (22). It is probable that overexpression overloads the retention capacity, and therefore the protein starts to be secreted into the medium. This was seen in microscopy by the appearance of fluorescent signals in the Golgi complex. A difference in the amount of secretion between insect cells and COS-7 cells can be explained by the different capacities of the cells to retain proteins in the endoplasmic reticulum, but it may be also due to the fact that the total expression of LH3/GGT per cell is higher in COS-7 cells than in insect cells.

The molecular weight of LH3 corresponds to about 85,000 in SDS-PAGE (8). GGT isolated from chick embryos has a molecular weight of 78,000 (13). It is not known if there are more than one GGT isoform. It is also not known if the molecular weight of GGT differs in different species. It is also possible that there is a difference in a glycosylation of the protein produced in the *in vitro* expression system compared with the protein isolated from tissues. Only one size protein is produced by LH3 cDNA in insect cells and COS-7 cells. Furthermore, only one protein is produced in a cell-free system supplemented with the LH3 cDNA sequence. In all these systems, the protein has the molecular weight of about 85,000, which corresponds to the molecular weight of LH3. These data indicate that no processing of LH3 is required to generate the GGT activity. Our data with anti-His-tag antibodies against the His tag present at the amino terminus of LH3 and with anti-GFP antibodies against the GFP tag present at the carboxyl terminus of LH3 do not indicate that there are multiple forms of LH3 in cells. Furthermore, our screening from different tissues does not give any evidence about alternative splicing for LH3 mRNA.

We have prepared polyclonal antibodies against a synthetic peptide corresponding to amino acids 283–297 of the human LH3 sequence (20), a region that is highly dissimilar between the lysyl hydroxylase isoforms. Antibody binding to that region of the LH3 molecule partially inhibited GGT activity. We also used antibodies that were prepared against GGT isolated as a homogenous protein from chick embryos. The binding sites of these antibodies are not known, but as observed earlier (19), these antibodies inhibited chick embryo GGT activity. The binding of the antibody partially prevented binding of the substrate, and the inhibition was more effective when tested with a high molecular weight substrate (19). When antibodies were tested with the enzyme produced by the human His-tagged LH3 construct in Sf9 cells, a similar inhibition of the GGT reaction was observed. The data obtained by LH3 and GGT
antibodies are in agreement with the suggestion that LH3 is identical to GGT. GGT antibodies furthermore suggest a similarity of binding sites of the antibodies between the chicken and human enzymes. These antibodies were also tested with GGT produced by human skin fibroblasts, and as seen in Table II, the LH3 and GGT antibodies showed similar inhibition of GGT under in vitro conditions.

Mutation analysis gave direct evidence that the amino acid sequence translated from the LH3 cDNA sequence is required and is sufficient for GGT activity. If we modified the sequence such that the molecule contained only amino acids 25–231 of the LH3 sequence, the protein has no GGT activity. This result indicates that the amino-terminal portion of the molecule was not sufficient to generate the GGT activity. Mutation of aspartate at position 669 to alanine totally eliminated LH activity. This change, however, has no effect or even a slightly stimulatory effect on the GGT activity, indicating that this amino acid residue is not involved in the catalytic process of the glucosylation reaction. The aspartate to alanine mutation has also been shown to reduce LH1 activity dramatically and indicates that aspartate 669 is a part of the active site of LH1 (23). LH1, LH2, and LH3 have a high homology in amino acid sequences; this holds true especially at the carboxyl-terminal region of the molecules (7–9). Our mutation analysis confirmed that the carboxyl terminus was the important region for the LH activity (5, 23). Interestingly, removal of the carboxyl-terminal end of the molecule does not totally eliminate the GGT activity, suggesting that the active center of GGT is not co-localizing with that of LH. This is also indicated by the finding that imidazole, a histidine analogue, effectively inhibited LH3 activity, whereas only a slight inhibition in GGT activity was observed. This may be explained by the finding that many conserved histidines in the carboxyl-terminal portion of the molecule are required for LH activity, as indicated in LH1 by in vitro mutagenesis (23), and imidazole probably prevents their participation in the catalytic process. Histidines do not appear to be necessary for GGT activity, however, and therefore imidazole does not inhibit GGT.

There is a 60% identity overall between the LH isoforms at the amino acid level (7–9). The carboxyl-terminal portion of the molecule is highly conserved in all three lysyl hydroxylases. In addition, there are regions in the middle of the molecule that have an identity of more than 80%. It is remarkable to find that only LH3, and not LH1, LH2a, or LH2b, possesses GGT activity. It is possible that regions having less identity between the LH isoforms, such as the amino-terminal end of the molecule and a region covering amino acids 271–356, are responsible for the GGT activity of LH3. More detailed studies are needed, however, to localize the amino acids of LH3, which contribute to the catalytic site of GGT.

CONCLUSIONS

Our results reveal the cDNA sequence of GGT, an enzyme catalyzing a unique post-translational modification of collagen. At the same time our data provide the first evidence for multifunctionality of the LH3 protein. The results are highly significant because they enable us to study in more detail the active site of collagen glucosyltransferase as well as the substrate requirements of the enzyme. In addition they facilitate the design of studies using transgenic animals to elucidate the functions of both the LH and GGT, which are disturbed in the animals, with altered levels of these activities and the cellular phenomena involved in glucosylation. These studies may also give indications of heritable disorders resulting from mutations, which limit or eliminate the glucosylation reaction in vivo.

Acknowledgments—We gratefully acknowledge Anna-Maija Koisti for expert technical assistance. Biocenter Oulu is acknowledged for excellent facilities in oligonucleotide synthesis.

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