Identification of Amino Acids Important for the Catalytic Activity of the Collagen Glucosyltransferase Associated with the Multifunctional Lysyl Hydroxylase 3 (LH3)*

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Collagen glucosyltransferase (GGT) activity has recently been shown to be associated with human lysyl hydroxylase (LH) isoform 3 (LH3) (Heikkinen, J., Risteli, M., Wang, C., Latvala, J., Rossi, M., Valtavaara, M., Myllylä, R. (2000) J. Biol. Chem. 275, 36158–36163). The LH and GGT activities of the multifunctional LH3 protein modify lysyl residues in collagens posttranslationally to form hydroxylysyl and glucosylgalactosyl hydroxylysyl residues respectively. We now report that in the nematode, Caenorhabditis elegans, where only one ortholog is found for lysyl hydroxylase, the LH and GGT activities are also associated with the same gene product. The aim of the present studies is the identification of amino acids important for the catalytic activity of GGT. Our data indicate that the GGT active site is separate from the carboxy-terminal LH active site of human LH3, the amino acids important for the GGT activity being located at the amino-terminal part of the molecule. Site-directed mutagenesis of a conserved cysteine at position 144 to isoleucine and a leucine at position 208 to isoleucine caused a marked reduction in GGT activity. These amino acids were conserved in C. elegans LH and mammalian LH3, but not in LH1 or LH2, which lack GGT activity. The data also reveal a DxD-like motif in LH3 characteristic of many glycosyltransferases and the mutagenesis of aspartate of this motif eliminated the GGT activity. Reduction in GGT activity was not accompanied by a change in the LH activity of the molecule. Thus GGT activity can be manipulated independently of LH activity in LH3. These data provide the information needed to design knock-out studies for investigation of the function of glucosylgalactosyl hydroxylysyl residues of collagens in vivo.

Glycosylation is a posttranslational modification important for the function of many proteins in vivo. For example, it is required for processes mediated by specific protein-carbohydrate interactions, such as protein targeting, cell adhesion, cell motility, protein dimerization, etc. (1). Mice carrying deletions in genes for glycosyltransferases have been described, some deletions leading to embryonic or perinatal lethality, whereas others are not essential for development and survival (1, 2).

Collagens are a large family of glycoproteins, which are ubiquitously distributed in almost all tissues of the body. It is known that collagens are structural building blocks of tissues and, in addition, have regulatory functions important for cell behavior. The biosynthesis of collagen is a multistep process including many posttranslational modifications, some of which are unique to collagens (3–6). These include the hydroxylation of lysyl residues and glycosylation of hydroxylysyl residues. The sugars linked to hydroxylysyl residues are galactose or a disaccharide glucosylgalactose and are present only in collagens and proteins having collagenous amino acid sequence in their structure (3–6).

The number of hydroxylysyl and glycosylated hydroxylysyl residues varies among different collagen types. The numbers may also vary in the same collagen type in different tissues and in different physiological states (3–6). Studies on bone collagen, for instance, indicate that the lysyl modifications vary among different skeletal regions according to the main function of the bone exerts in different locations (7). Hydroxylysyl residues play an important role in collagen cross-link formation, hydroxylysyl derived cross-links being typically found in skeletal and major internal connective tissues (3, 6, 8). Many of the cross-links are glycosylated (9). Recent studies have demonstrated changes in the cross-link profile in osteoporosis, a disease with a decrease in bone strength (8). There is evidence that hydroxylation of lysyl residues in collagen is associated with mineralization of fibrils, a high hydroxylation level preventing the deposit of minerals between the fibrils (10). Furthermore, studies on fibrillar collagens have indicated that an increased content of glycosylated hydroxylysyl residues of collagen is associated with a decrease of fibril diameter both in vivo and in vitro (11, 12). The detailed biological functions of galactoethylhydroxylysyl and glucosylgalactosylhydroxylysyl residues are not known, however. There are no studies so far using transgenic animals to study the consequences of abnormalities of the glycosylated hydroxylysyl residues in vivo, and there is no information regarding possible heritable disorders caused by defects in these modifications.

Lysyl hydroxylase (LH, EC 1.14.11.4) in the mouse and human is present in three different molecules, LH1, LH2, and LH3, originating from three different genes (16–21). Galacto-ethylhydroxylysyl glucosyltransferase (GGT, EC 2.4.1.66) catalyzes a transfer of the glucose from UDP-glucose to galactosylhydroxylysyl residues in the presence of Mn2+ (3–6, 13). We recently found (14) that GGT activity is associated with the

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† The abbreviations used are: LH, lysyl hydroxylase; GGT, galacto-ethylhydroxylysyl glucosyltransferase; DTT, dithiothreitol.
lysyl hydroxylase 3 (LH3) gene product. Accordingly, the first and last of three consecutive steps in the synthesis of hydroxylysine-linked carbohydrates in collagens are catalyzed by the same enzyme.

The ability to selectively delete or alter predetermined genes in an animal model provides enormous power for the study of gene function. The deletion of the mouse or human LH3 gene, for instance, will lead to a disturbance of the consecutive steps in hydroxylysine-linked carbohydrate formation. To investigate the specific function of glucosylation, however, the selective inhibition of the GGT activity of LH3 is required. To prepare for these studies, more information about the amino acids of LH3 responsible for the GGT activity is needed. In this study we have investigated the catalytic properties of the single ortholog of lysyl hydroxylase found in the nematode Caenorhabditis elegans (22) and found that LH and GGT activities are associated with the same gene product. We have characterized amino acids important for GGT activity in human LH3 and C. elegans LH and shown that these amino acids are localized at the amino-terminal part of the molecule, separate from the lysyl hydroxylase active site. This enables us to manipulate the gene to remove the GGT activity without affecting the LH activity of the gene product.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**Sf9 (Spodoptera frugiperda) insect cells were grown in SF-900 II serum-free medium (Invitrogen). Escherichia coli XL1-Blue cells transformed with different LH constructs were grown in LB broth containing 100 μg/ml ampicillin.

**Expression of cDNAs in Insect Cells—**Baculovirus transfer vector pFastBac1 in the BAC-TO-BAC™ (Invitrogen) expression system was used in the production of recombinant proteins. The LH3 construct (18) expressed in insect cells produced a recombinant protein having a signal peptide from the bacterial sequence and a His tag at the amino terminus after signal peptide cleavage, and the amino acids encoded by nucleotides 289–2455 of the LH3 cDNA sequence. The insect cells were harvested 72 h after infection and homogenized as described earlier (18).

**Expression of cDNAs in Bacterial Cells—**The LH3 cDNA construct used in this study was described elsewhere (14). The insert for the C. elegans LH (corresponding to amino acids 14–730 in the cDNA) was generated by PCR (Dr. Robert Barstead kindly provided us with a C. elegans cDNA library) and ligated into the E. coli StuI-PstI site of a pQE30 vector. The recombinant proteins have the whole coding sequence without the signal sequence and a His tag at the amino terminus with a pQE30 vector. The recombinant proteins have the whole coding sequence generated by PCR (Dr. Robert Barstead kindly provided us with a C. elegans cDNA library) and ligated into the E. coli StuI-PstI site of a pQE30 vector. The recombinant proteins have the whole coding sequence without the signal sequence and a His tag at the amino terminus with a pQE30 vector.

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**Enzyme Activity Assays—**LH activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate (23) with the synthetic peptide IKGIKIHKG as a substrate. GGT activities were measured by a method based on the transfer of radioactively (tissue) labeled glucose from UDP-sugar to galactosylhydroxylysyl residues in a calf skin gelatin substrate (24). The specific detection of the reaction products was performed after alkaline hydrolysis.

**Other Assays—**In vitro translation was carried out as described elsewhere (14) by using a pCITE 4a vector (Novagen) for the transcription and a STP3 kit (Novagen) for the translation. Western blot analysis was carried out using monoclonal antibodies against the His tag (Sigma). The proteins were fractionated under reducing or non-reducing conditions by 10% SDS-PAGE, blotted onto an Immobilon-P membrane (Millipore), and incubated with the primary antibody. Anti-mouse IgG peroxidase conjugate (Zymed Laboratories Inc.) was used as the secondary antibody. Bound antibodies were visualized using the ECL detection system (Amersham Biosciences) and x-ray film (Eastman Kodak Co.). The QuikChange site-directed mutagenesis kit (Stratagene) was used to make mutations in the cDNA sequences. The nucleotide changes of the mutations were confirmed by sequencing.

**RESULTS AND DISCUSSION**

**GGT Activity in E. coli Cells Transformed with the C. elegans Lysyl Hydroxylase cDNA Construct—**The whole genome of C. elegans has been recently sequenced (15), and the sequence data indicate that there is a single gene for lysyl hydroxylase (22). To express the protein in E. coli and to determine whether the LH gene product possesses the GGT activity, which has been found to be associated with human LH3 (14), we have prepared a cDNA construct for C. elegans LH. Control E. coli cells, containing only the pQE30 vector, as well as cells expressing DHFR, have only very low residual GGT activity (<300 dpm/mg soluble cell protein). When the C. elegans LH cDNA was expressed in E. coli cells, GGT activity was present in the soluble protein fraction. Due to different expression levels, the activity varied in different experiments, giving values in the range of 21,000 up to 30,700 dpm/mg soluble protein.

**Amino Acids Conserved in LH3 and C. elegans—**Our data indicate that GGT activity is associated with the LH of C. elegans and LH3 of human (14) and mouse, whereas human LH1 and LH2 possess only LH activity (14). To determine the conserved amino acids for the multifunctional LH/GGT protein (Fig. 1) we have aligned the C. elegans LH and mouse and human LH3 cDNA sequences and compared them to the sequences of LH1 (bovine, rat, chicken, mouse, human) and LH2 (mouse, human). There are 29 amino acids conserved between the LH3 sequences and C. elegans sequence (LH3-C. elegans-specific amino acids) but differing from the LH1 and LH2 sequences (shaded black, Fig. 1). The amino acids are scattered evenly throughout the molecule. The whole Drosophila genome has also recently been sequenced (25), and only one gene was found for LH in this genome. As seen in Fig. 1, 11 of the 29 amino acids are conserved also in the Drosophila lysyl hydroxylase.

**LH3-C. elegans-specific Amino Acids Necessary for GGT Activity—**In vitro mutagenesis combined with an in vitro translation system was used in our experiments (Fig. 2). Both C. elegans and human LH3 sequence were used in the studies. We started to search the important amino acids for GGT activity with C. elegans LH. The most inhibitory changes and one as a control of carboxyl-terminal area were tested further with human LH3. Aspartic acid at position 392 in human LH3 conserved in all species of LH isoforms was mutated to alanine as it was one candidate of the DXD motif of the molecule (see later). The data was then confirmed in an E. coli system for the human LH3 (not shown). We mutated the LH3-C. elegans-specific amino acids to the amino acids present in LH1 and/or LH2. As seen in Fig. 2, mutations at the amino-terminal end of the molecule caused an inhibition of GGT activity, whereas mutations at the carboxyl-terminal end of the molecule had no effect on this activity. Two mutations, the C144I and L208I in the human sequence increased GGT activity. Furthermore, a mutation of Ala®64 in the C. elegans sequence, inhibited GGT activity markedly in both species suggesting that these amino acids, conserved in LH3-C. elegans LH, are important for the catalytic activity of GGT. Our data also reveal some differences in the GGT active sites between C. elegans and human. Two leucine changes, L124V and L125V, in the C. elegans sequence inhibited the GGT activity by 40 and 70%, whereas the corresponding mutations in the human sequence increased GGT activity. Furthermore, a mutation of A453I in the C. elegans sequence (corresponding to Ala®64 of the human LH3 sequence) caused a remarkable inhibition of GGT activity, whereas the corresponding change in

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2 L. Sipilä, J. Heikkinen, and R. Myllylä, unpublished data.
FIG. 1. Alignment of lysyl hydroxylase isoform sequences of bovine, rat, chicken, human, mouse, C. elegans, and Drosophila. Bovine (GenBank™ accession no. AF054274), rat (GenBank™ accession no. X74879), chicken (GenBank™ accession no. M59183), mouse (GenBank™ accession no. AF046783), and human (GenBank™ accession no. AF046889) sequences most probably represent the LH1 isoform of lysyl hydroxylase. Only one isoform for lysyl hydroxylase is found in the genomes of C. elegans (GenBank™ accession no. Z66512) and Drosophila (GenBank™ accession no. AE003545). The amino acids conserved between the LH3 sequences (mouse, GenBank™ accession no. AF046782; human, L06419) and the C. elegans sequence but differing from LH1 (mouse, GenBank™ accession no. AF046782; human, L06419) and LH2 (mouse, GenBank™ accession no. AF046572; human, U84573) sequences are shaded black, whereas DXD-like motifs in these molecules are shaded gray.

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the human sequence had no effect on this activity.

During in vitro mutagenesis studies we also generated a *C. elegans* molecule, in which Leu\(^{124}\) (*C. elegans* sequence) was mutated to valine, and the molecule also contained a tandem duplication of 11 amino acids (His-Tyr-Ser-Glu-Lys-Arg-Val-Leu-Phe-Gly-Ala) in the amino-terminal region. This change reduced the GGT activity dramatically when compared with a single amino acid change in the molecule, giving a GGT activity value of 0.5% (not shown). These data suggest that disruption of the structure of the molecule by the insertion of amino acids at this position abolishes the GGT activity.

There is no data about the multifunctionality of the LH gene in *Drosophila*, but it is interesting to note that the conserved amino acids, Cys\(^{144}\) and Leu\(^{208}\) in the human sequence, are also found in the *Drosophila* LH, suggesting a possible association of GGT activity with the insect LH gene. It is worth noting that the amino acid corresponding to Ala\(^{464}\) of the human sequence (Ala\(^{453}\) in *C. elegans* sequence) is isoleucine in

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**Fig. 2. The effect of mutation of LH3-*C. elegans*-specific amino acids on GGT activity.** *C. elegans* LH and human LH3 were expressed by an in vitro translation system. The translation level was analyzed by SDS-PAGE and autoradiography (not shown). The activity without any mutation is taken as 100.
the Drosophila sequence, which corresponds to the LH1 sequence in this position.

Cys44 Forming a Disulfide Bond in LH3 Structure?—We used the alkylation agent, iodoacetamide, to block free sulfhydryl groups to determine whether the human Cys44 forms a disulfide bond in the LH3 molecule (26). E. coli cells producing His-tagged LH3 were lysed in lysis buffer (see “Experimental Procedures”) in the presence of 10 mM iodoacetamide. The LH3 was purified with a nickel column and treated with or without 125 mM DTT before separation by 10% SDS-PAGE. The mobility of LH3 was recognized on the Western blots by a His tag antibody. Lanes 1 and 2: LH3 without mutation. Lanes 3 and 4: LH3 with Cys44 mutated to Ile. Lanes 5 and 6: LH3 with Cys44 mutated to Ser. Lanes 7 and 8: LH3 with Cys44 mutated to Ser. The presence and absence of DTT is indicated.

The Amino Acid Changes Inhibiting Glucosyltransferase Activity Do Not Affect LH Activity—The baculovirus expression system was used to determine whether amino acid changes that inhibited the GGT activity of LH3 have an effect on the LH activity. We studied the most effective mutations of human LH3, i.e. C144I and L208I, in an LH activity assay. Our results (data not shown) indicate for GGT that the results were independent on the expression system used for the recombinant protein production, the baculovirus, and in vitro translation, and that the E. coli system gave similar values for the mutated molecule. Our data for LH indicates that these mutations did not have any effect on LH activity, however. This is consistent with our earlier data obtained from LH1, that the amino acids responsible for LH activity are located in the carboxyl-terminal portion of the molecule (27, 28). The carboxyl-terminal part of the molecule is the most conserved region among the LH isoforms (16–20).

The Amino-terminal Part of LH3 Is Able to Generate GGT Activity—Our data above suggest that the amino-terminal portion of the LH3 molecule is important for GGT activity. To determine whether the carboxyl-terminal portion of the molecule is required for GGT activity, we removed amino acids from the carboxyl-terminal end of the human LH3. We generated a translational stop codon in different parts of the molecule, and the truncated LH3 molecules produced in E. coli and insect cells (baculovirus system) were assayed for GGT activity (Fig. 4). These data indicate that shortening the carboxyl-terminal portion of the LH3 molecule reduces, but does not eliminate, GGT activity. A 355-amino acid-long portion of amino-terminal moiety is still able to generate a low GGT activity (Fig. 4). None of the truncated molecules were able to hydroxylate lysyl residues (not shown), confirming our previous finding that amino acids important for lysyl hydroxylase activity are located on the carboxyl-terminal portion of the molecule (27, 28).

A DXD Motif Required for GGT Activity—Sequence alignments and x-ray crystal structures have revealed a so-called DXD motif in many glycosyltransferases, and this motif has been found in different glycosyltransferase families (29). The motif is thought to stabilize the Mn2+ and thus indirectly stabilize the binding of the diphosphate moiety of the UDP-sugar (29, 30). Therefore we looked for this motif in LH3. There are at least three DXD-like motifs in the LH3 sequence (Fig. 1). Our data from the first screenings, a point mutation of DAD to alanine, and the GGT activity was measured. The expression level of recombinant protein was analyzed by His tag antibodies on Western blot as shown in the figure.
A sequence, Cys144, which is probably not disulfide-linked, and a lysyl hydroxylase isoform (18, 19). In the human LH3 associated with a single human gene product (14), which is also section of collagens. The activity has recently been found to be ferase associated with unique hydroxylysine-linked glycosyla-

for collagen glucosyltransferase activity, the glycosyltrans-
both molecules.

identical in these species although GGT activity is present in
observed in our data, there are evolutionary changes also be-
diverged during evolution from the ancestral LH and LH3 (20)
proteins were expressed in LH1/LH2, see Fig. 1), and Ile to Leu (corresponding to the change L208I between LH3 and LH1/LH2a/LH2b, see Fig. 1) in LH1 and LH2. We also generated molecules where both changes were introduced in the same molecule. The mutated proteins were expressed in E. coli, and GGT activity was measured. However, the activity measurements revealed no GGT activity (not shown). This data suggest that LH1 and LH2 have diverged during evolution from the ancestral LH and LH3 (20) such that replacement of the two most conserved amino acids into their structures is not sufficient to restore GGT activity. As observed in our data, there are evolutionary changes also be-

between LH3 and C. elegans sequence; the active sites are not identical in these species although GGT activity is present in both molecules.

Conclusions—We report in this paper amino acids important for collagen glucosyltransferase activity, the glycosyltrans-
ferase associated with unique hydroxylsine-linked glycosyla-
tion of collagens. The activity has recently been found to be associated with a single human gene product (14), which is also a lysyl hydroxylase isoform (18, 19). In the human LH3 sequence, Cys144, which is probably not disulfide-linked, and Leu208 are important for GGT activity. These amino acids are also found in the C. elegans LH gene product, which possesses GGT activity. The corresponding amino acids in LH1 and LH2a/LH2b are isoleucine, and neither isoform has GGT activity. Mutation of the cysteine to isoleucine and the leucine to
iso leucine in LH3 reduces GGT activity dramatically. These conserved amino acids are not responsible for the LH activity of LH3, suggesting that the LH and GGT active sites are separate on the LH3 molecule. The distinct locations of the active sites of LH and GGT on the LH3 molecule were also demonstrated by truncation of the LH3 molecule, which removed the LH active site. The truncated molecules still retained some GGT activity. Our data furthermore reveal a DXD-like sequence, a motif characteristic of many glycosyltransferases, required for GGT activity in the multifunctional LH molecule.

The results of this study are highly significant. We have identified amino acids important for GGT activity, which provide tools to manipulate this activity of the LH3 molecule. This knowledge is essential for the design of knock-out and transgenic studies to elucidate the functions of the glucosylation of galactosylhydroxylysyl residues in vivo. These experiments may also provide information about possible heritable disor-
ders associated with abnormal glucosylation of galactosyl-
hydroxylysyl residues in vivo.

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