Nine of Ten Histidine Residues, Located in Three Conserved Clusters, are Required for Mammalian Sterol C5-Desaturase Activity*

Hideaki NISHINO[§], Shinya NISHI and Teruo ISHIBASHI[†]

Department of Molecular Biochemistry, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

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ABSTRACT

We previously reported three histidine-rich conserved motifs containing HX3H, HX2H and HX2HH in the deduced primary amino acid sequence of human sterol C-5 desaturase (SC5D), which introduces a double bond at the C5, 6 positions of Δ^7 -sterols to form $\Delta^5,7$ -sterols (Nishi, S. *et al.* (2000) *Biochim. Biophys. Acta* 1490, 106–108). In the present study, the role of the 18 His residues contained in the enzyme was investigated by means of site-directed mutagenesis and expression of the mutated enzymes in an SC5D-deficient yeast strain (erg3). The activity of the recombinant SC5Ds to allow the yeast to convert ergosta-7,22-dien-3 β -ol to ergosterol (ergosta-5,7,22-trien-3 β -ol) was examined directly in the erg3 strain *in vivo*.

Individual replacements of each of the ten conserved His residues with Arg completely eliminated the desaturase activity in each case, except for one, His190. The conserved His-containing clusters have a consistent positioning with respect to the putative membrane spanning domains, and we propose that they are located on the cytoplasmic face of the endoplasmic reticulum. In contrast, mutation of eight nonconserved flanking His residues to Arg did not affect the ability to complement the erg3 phenotype. Therefore, these non-conserved His residues are not essential for the catalysis, but may contribute to the activity through conformational or other effects.

Western blot analysis confirmed that the steady-state expression levels were equivalent for the wild-type human SC5D and all the mutants, supporting the idea that the conserved His residues are essential for catalytic function. A possible role for these His residues could be to act as ligands for the iron atom(s) contained in the enzyme.

Hydropathy analyses indicated that the enzyme contains at least four membrane-spanning domains. Topologically, SC5D is predicted to have a C-terminal cytoplasmic domain and an N-terminal region located in the luminal side of the endoplasmic reticulum, since the N-terminal sequence was resistant to proteinase K proteolysis, but the C-terminal sequence was not.

*Data deposition: The sequences reported in this paper have been deposited in the DDBJ database (accession numbers: AB016247 for human sterol-C5-desaturase mRNA, AB016248 for mouse sterol-C5-desaturase mRNA, and AB052846 for rat sterol-C5-desaturase mRNA).

[§]Present address: Liberal Arts (Chemistry), Sapporo School of The Arts, 1 Geijutsuno-mori, Minami-ku, Sapporo 005-0864, Japan

[†]To whom correspondence should be addressed: Dr. Teruo Ishibashi, Department of Molecular Biochemistry, Hokkaido University Graduate School of Medicine, N15W7, Sapporo 060-8638, Japan
Tel: +81-11-706-5047, Fax: +81-11-706-5169
E-mail: t-ishiba@med.hokudai.ac.jp

Sterols are essential cellular molecules which function to regulate membrane fluidity, permeability, the activity of membrane-bound enzymes and the cell growth rate (4). All the enzymes required for the synthesis of cholesterol from squalene, the first sterol intermediate, are bound to the endoplasmic reticulum (4), which can be isolated from cell-free homogenates as microsomes. However, it is difficult to isolate individual microsomal enzymes from cholesterologenic tissues and organs. Sterol C5-desaturase (SC5D) is the terminal enzyme system that introduces a double bond at the 5,6 positions of Δ^7 -sterols to form $\Delta^5,7$ -sterols (26). $\Delta^5,7$ -Sterols are reduced to produce Δ^5 -sterols as the final form in higher eucaryotes. SC5D is a multicomponent enzyme system which is cyanide-sensitive, requires iron and molecular oxygen and is coupled to NADH-cytochrome b5 reductase and cytochrome b5 in the microsomal electron transport system (10).

We have tried to solubilize and purify mammalian SC5D from liver microsomes (10) and we studied the enzyme kinetic properties when the enzyme and the substrate are present in the same membrane (12, 33). However, purification of the enzyme was very difficult because of its instability after solubilization. Consequently, the nature of the iron-containing active site in the membrane desaturase is poorly defined. cDNAs homologous to fungal (2, 7) and plant (34) SC5D have been cloned from libraries constructed from human fetal brain and adult brain mRNA (19), but we isolated another sequence clone from an adult human cDNA library (21). Mouse and rat cDNAs homologous to our human base sequence, but not to the previously reported one, were also cloned from libraries (21). The high degree of evolutionary conservation of amino acid sequences in the central portion suggests that the product of the SC5D gene may serve an important function in the cholesterol synthesis pathway in humans. Interestingly, the protein is expressed in brain, heart, spleen, liver, and kidney, but not in leucocytes. Alignment of the deduced amino acid sequences revealed the following features: (i) four putative membrane-spanning regions, together with a cytoplasmic domain; (ii) three His-rich motifs (H138X3H, H151X2H, H229X2HH); (iii) a conserved region of ten His residues whose function is unknown.

The enzyme may contain non-heme iron(s) in the catalytic center in a histidine-rich region, because, firstly, the enzyme can be inhibited by cyanide ions and Tiron (10), and secondly, the other desaturases, acyl-CoA oxidoreductases, hold non-heme iron in the active center, which also contains phylogeneti-

cally conserved His residues (5).

Expression of the human SC5D gene in a desaturase-deficient mutant of *Saccharomyces cerevisiae* (erg3) complements the mutation. This complementation provides a method to assess the functional significance of the conserved His. In this communication, we describe a study of the functional role of the conserved His residues, as assessed in terms of the ability of the human SC5D gene, after site-directed mutagenesis, to replace the deficient gene function in erg3.

MATERIALS AND METHODS

Materials. A Superscript human liver cDNA library was obtained from Gibco (Maryland, USA), a mouse liver 5-stretch plus cDNA library from Clontech (California, USA), and PCR amplification kit from Takara (Shiga, Japan). The TA cloning kit dual promoter ver. A, a yeast expression vector, pYES2, which carries a yeast origin of replication, the URA3 selection marker, an expression cassette based on the GAL1 portion of the divergent GAL1/GAL10 promoter region from *Saccharomyces cerevisiae* for galactose-inducible expression of genes inserted into the polylinker, a CYC1 transcription termination signal, the 2 m origin of replication, a partitioning element from YEp24 and the URA3 gene for selection in yeast ura3-mutant, and *Saccharomyces cerevisiae* strain INVSC2 (MAT α , his3- Δ 200, ura3-167) were purchased from Invitrogen (California, USA). The ABI prism BigDye terminator cycle sequencing ready reaction kit was from Applied Biosystems (California, USA), T7 sequenase ver. 2.0 DNA sequencing kit, 35 S-dATP and 32 P-dATP from Amersham (Ohio, USA), and all other chemicals from Wako (Osaka, Japan) and Sigma (Missouri, USA).

PCR. Nucleotide sequences in partial human cDNA clones (GenBank accession no. F11632) and partial mouse cDNA clones (W65233) homologous to the erg3 SC5D from *Saccharomyces cerevisiae* (P32353) were identified with the algorithm of the SIM-Local alignment tool for nucleic acids (11). As a result, the primers HU-214 TCACTGACATGTTTCATCTACTG GAT for the sense strand, and HU-215 CACGGTCA TGAATGGAAATTGTCC for the antisense strand, were added to the amplification reaction mixture, which contained a human liver cDNA library as a template. The thermal cycler was programmed to perform 1 cycle of 91°C for 5 min and 50°C for 10 min and 30 cycles of 94°C for 1 min, 50°C for 2

min, and 72°C for 2 min. The PCR product, 249 bp, was labeled with ^{32}P for use as a probe for screening human liver cDNA libraries. DNA sequencing was performed on both strands according to the BigDye terminator cycle sequencing procedure or by using ^{35}S -dATP. Alignments and hydrophobicity analysis were calculated with the algorithms of the SIM alignment tool for proteins (11) and according to Kite and Doolittle (14), respectively.

Expression in *Saccharomyces Cerevisiae* and Detection of SC5D Activity. Protein expression in *Saccharomyces cerevisiae* was performed as described (16). The 5'-noncoding region of human SC5D cDNA was replaced with oligonucleotide AAAGAATTCATGGATCTTGTACTCCGTG, introducing 5' EcoRI restriction sites for subcloning in the yeast expression plasmid pYES2. Lithium acetate transformation of yeast strain INVSC2 was performed as described (12). Concentrations of proteins were determined by the method of Lowry *et al.* (18) with bovine serum albumin as the standard. Expression of the SC5D ORF was verified by immunostaining using rabbit antibody raised against the anti-N-terminal region oligopeptide (YPATWPEDDIFRQA) or anti-C-terminal region oligopeptide (KNEKLFNGEFTKTE). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli (15) on a 12.5% polyacrylamide gel. The enzyme activity of the heterologously expressed SC5D was determined from the assay of conversion of ergosta-7,22-dien-3 β -ol to ergosterol (ergosta-5,7,22-trien-3 β -ol) using reversed-phase high-pressure liquid chromatography, as described previously (33).

Yeast Cell Culture. Yeast cells transformed with pYES2 and pYES2/human SC5D were grown to the stationary phase in SGRI synthetic medium. The saturated culture was diluted with an equal volume of YP medium supplemented with ethanol (final concentration 1.5% by volume) as a carbon source and grown until the cell density reached a minimal value of 7×10^7 cells/ml ($A_{600} = 10$). SC5D expression was induced by addition of D-galactose at a final concentration of 20 g/liter. The culture was terminated when the cell density reached 1.5 to 2×10^8 cells/ml ($A_{600} = 20$ –30).

Site-Directed Mutagenesis. The mutated alleles of SC5D were obtained by the introduction of point mutation using a Transformer site-directed mutagenesis kit (2nd ver., Clontech) (California, USA), with the primers shown in Table 1. All the mutations

were verified by determination of the sequences of mutagenized regions by the dideoxy chain-termination method (27), using a sequencing kit (T7 Sequenase ver. 2.0).

RESULTS

Prediction of Transmembrane Topology of Human Sterol C5-Desaturase

We propose a model in which the protein has four hydrophobic domains, each capable of spanning the membrane and arranged such that the three hydrophilic domains (containing the conserved His residues) reside at the cytoplasmic face of the endoplasmic reticulum. A diagram of this arrangement is shown in Fig. 1. The conserved His-containing regions are uniformly predicted to be in hydrophilic regions of the primary sequence, rather than in membrane-spanning domains, and each of these regions is predicted to reside on the cytoplasmic face of the membrane (Fig. 2). H138X3H is located near the transmembrane region, H151X2H lies between transmembrane domains, and H229X2HH is near the carboxyl terminus of the peptide.

In addition to the hydropathy algorithm (14), the distribution of strongly basic amino acids, Lys and Arg, helps predict possible transmembrane configuration of integral membrane proteins, based on the "positive inside rule" (37). The transmembrane region I is thought to be a type I signal anchor sequence because of the rare N-terminal positively charged amino acids and the following Leu-rich hydrophobic stretch possessing sufficient length to span the membrane α -helix (17, 31, 38).

Since the substrates of SC5D are highly hydrophobic, they will probably partition into the lipid bilayer. In contrast, electron transfer for the enzyme occurs on the cytoplasmic side in the microsomal membranes (32). These two properties suggest that an active site assembled from these His-containing sequences may lie at or near the membrane surface.

Functionality of the Conserved His Residues of SC5D Assessed by Site-Directed Mutagenesis

We previously isolated cDNA of human SC5D containing an ORF coding for a protein with 299 amino acid residues (33). A noteworthy feature of the SC5D is the presence of three His-rich motifs, and the nucleotide sequence predicts the presence of 18 histidines.

In order to investigate the role of the conserved His residues in human SC5D, we have made use of the ability of the desaturase gene to complement the

Fig. 1 Hydropathy profile of human SC5D. On the ordinate, hydrophobicity is indicated by positive numbers.

Fig. 2 Proposed topological model of the human SC5D protein and effect of mutagenesis on the enzyme activity.

erg3 mutation in *Saccharomyces cerevisiae* (Fig. 3). By using a polymerase chain reaction-based site-directed mutagenesis strategy, we examined the effect of replacement of the ten conserved and eight non-conserved His residues with sterically conservative Arg residues, which are electrically positive, like His.

The effects of these mutations on the *in vivo* activity of human SC5D expressed in the *erg3* null mutant cells are shown in Table 1. The mutation of any of 9 of the 10 conserved His residues with Arg totally eliminated the desaturase activity. In contrast, mutation of the eight nonconserved flanking His residues to Arg did not affect complementation of the *erg3* phenotype. These findings suggest that the conserved His residues, except for H190, are all essential for the enzyme activity.

Based on the propensity of His residues to serve as metal ligands, one possibility is that some or all of these His residues serve as iron ligands. The presence of closely spaced His residues is consistent with chelate binding models that increase the affini-

ty for transition metals by up to three orders of magnitude relative to a single His (1). Thus, these results strongly support the assignment of the conserved three His motifs, containing HX3H, HX2H, and HX2HH sequences, in the membrane desaturases as iron ligands.

Heterologous Expression of Mutated SC5D cDNAs

In order to assess whether the His to Arg mutation inhibits enzyme protein synthesis via changes in mRNA or protein stability, immunostaining of total yeast cellular extracts was performed using anti-C terminal region peptide antibodies as a probe (Fig. 4A). Total yeast cellular extract lacking the human SC5D gene showed no immunoreactivity, whereas all other lanes, containing the site-directed mutant forms of the ten conserved His, showed equivalent levels of immunostaining without any marked over-expression. Therefore, the non-complementation of desaturase-deficient yeast cells after mutations of conserved His residues can not be attributed to a lack of protein expression.

Table 1 Synthetic oligonucleotide primers used for site-directed mutagenesis and the SC5D activities expressed in the mutants

His residue	Conservation	Oligonucleotide sequence ^a (5'-3') (sense)	Complementation after mutation
60	No	GTCTTCGATCGTGCATTAATG	Yes
65	No	GCATTAATGAAACGTCCACAAT	Yes
106	No	AGCAAATTACGTGATGACCTA	Yes
138	Yes	ATCTACTGGATTTCGCAGAGGCCTTCATC	No
142	Yes	AGAGGCCCTTCGTCATAGACTG	No
143	No	GGCCTTCATCGTAGACTGGTA	Yes
151	Yes	AAGCGCCTACGTAAACCTCACCATATTTG	No
154	Yes	CATAAACCTCGCCATATTTGG	No
155	No	AAACCTCACCGTATTTGGAAG	Yes
166	No	ATTTGCAAGTCGTGCTTTTCAC	Yes
169	Yes	TCATGCTTTTCGCCCTATTGATGGCTTTC	No
181	No	CTACCTTACCGTATATACCCT	Yes
190	Yes	ATCTTTCCATTACGCAAGGTGG	Yes
209	Yes	ACAATTTCATTTCGTGACGGTG	No
228	No	TGGCTCAGCTCGTCATACAGAC	Yes
229	Yes	TCAGCTCATCGTACAGACCACC	No
232	Yes	TCATCATACAGACCGCCATATGTTC	No
233	Yes	TCATACAGACCACCGTATGTTCTTTG	No

^aCodons for the changed amino acids are underlined. Nucleotides represented in bold character G is substituted from A.

Fig. 3 *In vivo* expression of human SC5D in *Saccharomyces cerevisiae* *erg3* mutant. After incubation of ergosta-7,22-dien-3 β -ol under the standard conditions, the reaction mixture was extracted and the extract was concentrated under nitrogen gas. An aliquot was injected into an analytical reversed-phase high-pressure liquid chromatograph. Ergosta-7,22-dien-3 β -ol and ergosterol (ergosta-5,7,22-trien-3 β -ol) were monitored at 210 nm and 280 nm, respectively.

Fig. 4 Heterologous expression of mutated cDNAs. (A) Immunostaining with antibody against the C-terminal region polypeptide. Antibodies were visualized with anti-rabbit IgG conjugated with horseradish peroxidase. For Western blotting, 5 μ g of yeast cell extract was separated on a 12.5% SDS polyacrylamide gel under reducing conditions (sample buffer containing 10 mM DTT) and immunostained with 20 ng/ml C-terminal peptide antibody. Lanes 1–10 contain the mutants of the ten conserved His residues, H138, H142, H151, H154, H169, H190, H209, H229, H232 and H233, respectively. (B) Immunostaining before (lane 1) and after (lanes 2, 3) proteinase K digestion of SC5D expressed in *erg3* yeast cells with antibodies against anti-C-terminal region oligopeptide (lanes 1, 3) and anti-N-terminal region oligopeptide (lane 2).

On the other hand, it has been demonstrated that the N-terminal of human SC5D is located on the luminal side of the endoplasmic reticulum, since the N-terminal sequence was resistant to proteinase K, whereas the C-terminal sequence was not (Fig. 4B).

DISCUSSION

Sterols are major components of eucaryotic cell membranes. End products of the sterol biosynthetic pathway differ depending on species; cholesterol is encountered in animals, and ergosterol is the most common sterol in fungi, while sitosterol is typical plant sterol (9). The enzymatic steps that allow conversion of lanosterol, generated by oxidative cyclization of squalene, to cholesterol have been well documented (13, 22, 23, 25, 29). In animals, the main pathway involves the following sequences: isomerization of the double bond from C-8 to C-7, introduction of a double bond at C-5, and two reduction steps at the C-24 and C-7 double bonds. The quantitative and qualitative sterol compositions and the presence of double bonds or branched groups are known to modulate membrane properties. This aspect has been well documented in fungi and especially in the yeast *Saccharomyces cerevisiae*, since various mutant strains are available (3, 20).

We previously isolated the SC5D gene, and found 10 conserved His residues in the predicted amino acid sequence (21). These conserved His residues were located within three conserved clusters, H138X3H, H151X2H and H329X2HH. These His-rich motifs are characteristic of many membrane-bound fatty acid desaturases, and reportedly bind non-heme iron that is required for enzymatic activity (30). Histidine, with its weakly basic imidazole group ($pK_a = 6.0$), can donate a proton to serve as a ligand to transition metals in the neutral pH range. In order to examine the importance of the His residues in SC5D, we used site-directed mutagenesis to replace each His residue individually with Arg, an amino acid that contains a strongly basic guanidium group ($pK_a = 12.5$). Then we analyzed the effects of the mutations on the activity of the enzyme (Table 1).

One could ask whether some of these mutated desaturases might interact differently with yeast cytochrome b5. It is difficult to answer this question directly, since we employed an *in vivo* functional yeast recombinant membrane-bound desaturation system and, in addition, the system was heterologous. For example, rat liver wild-type and mutant cytochrome b5 proteins have recently been function-

ally characterized using the yeast cytochrome b5 reduction system (36). The results for a number of mutated acyl-CoA desaturases show the limitations of a method consisting of choosing evolutionarily conserved residues to identify catalytically essential residues based solely on the molecular evolution of a group of enzymes.

To obtain further information about the characteristics of the various mutant desaturases, such as their iron content and the impact of each mutation on the physical and biochemical features of the enzyme, the next and crucial step is purification, without loss of activity, of the enzyme from yeast cells that have been transformed with the gene for each of the mutated desaturases. In the absence of structural information about this class of membrane-bound desaturases, it is difficult to speculate about the precise roles of the mutated residues. It would be useful to develop an expression system which would allow a thorough enzymological and structural study of the enzyme. For example, in spite of intensive investigations leading to a variety of proposals (8, 24, 28), the precise mechanism of SC5D has not been elucidated so far, especially with regard to the question of whether C5(6)-desaturation involves a C5- or C6-hydroxylated intermediate (6, 24, 28) or a direct dehydrogenation process (8, 35).

In conclusion, the use of *in vivo* assay to analyze the activity of mutant SC5D in this work has opened a door to understanding the structure-function relationship of this membrane-bound desaturase. The precise mechanism of the catalytic reaction and the nature of the involvement of the active-site residues will need further investigation. The present work also demonstrates that it is possible by site-directed mutagenesis to increase the activity of an enzyme involved in the postqualene phase of sterol biosynthesis.

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