Mutagenesis of putative serine–threonine phosphorylation sites proximal to Arg255 of human cytochrome P450c17 does not selectively promote its 17,20-lyase activity

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Objective: To investigate the role of serine–threonine phosphorylation on the activity of human P450c17.
Design: In vitro study.
Setting: Academic basic research laboratory.
Patient(s): None.
Intervention(s): P450c17 expression constructs with a FLAG-tag on either the C-terminus or N-terminus of the protein were generated. Human C-terminal FLAG-tagged P450c17 chromosomal DNA was subjected to site-directed mutagenesis. Serine 258 and threonine 260 each were mutated to alanine and aspartic acid. The mutant P450c17s were expressed in COS-7 cells, and the enzymatic activities were measured.
Main Outcome Measure(s): 17α-Hydroxylase and C_{17-20} lyase activities of human P450c17.
Result(s): C-terminal FLAG-tagged P450c17 functioned indistinguishably from the wild-type P450c17. Mutants S258A, S258D, and T260D had significantly less 17α-hydroxylase and C_{17-20} lyase activities than the wild type.
Conclusion(s): Adding an epitope tag to the C-terminus of the P450c17 protein does not interfere with its activities and will be a useful tool to isolate human P450c17 protein from cultured cells. Phosphorylation of serine 258 but not threonine 260 may act as a physiologic regulator of both enzymatic activities through interaction with obligatory redox partners. (Fertil Steril® 2006;85(Suppl 1):1290–9. ©2006 by American Society for Reproductive Medicine.)

Key Words: P450c17, 17α-hydroxylase, C_{17-20} lyase, androgen production

In human steroidogenic tissue, cytochrome P450c17, encoded by a single gene located on chromosome 10q24.3 (1–5), is the sole enzyme that catalyzes both 17α-hydroxylation and C_{17-20} lyase conversion of C_{21} steroids to C_{19} androgen precursors (6–10). P450c17 thus plays a key role in the biosynthesis of androgenic hormones (11). The P450c17 gene is hormonally induced, and regulated developmentally, and in a tissue-specific manner in both the adrenals and the gonads (2, 4, 6, 12–17).

Similar to other microsomal cytochrome P450s, the catalytic activities of P450c17 are particularly dependent on electron donation from nicotinamide-adenine dinucleotide phosphate (NADPH), a reaction mediated by membrane-bound, electron transfer flavoprotein P450 oxidoreductase (OR) (18, 19). The 17α-hydroxylase and C_{17-20} lyase activities of P450c17 are regulated at the posttranslational level by the proper abundance of P450 oxidoreductase (20, 21) and by the action of cytochrome b_5, which allosterically promotes interactions with OR (22–25).

Recent observations also suggest that the ratio of hydroxylase–lyase also may be governed by the phosphorylation of P450c17 itself (26, 27). In the absence of phosphorylation, CYP17 possesses full 17α-hydroxylase activity but very little C_{17-20} lyase activity (27, 28). Evidence in the literature suggests that posttranslational phosphorylation of P450c17 on serine and threonine residues selectively increases the C_{17-20} lyase activity without significantly altering its 17α-hydroxylase activity or altering substrate binding to the active site of the enzyme (11, 28).

All mutations proven to directly and selectively impair the C_{17-20} lyase activity of human P450c17 involve residues that lie either within the active site or adjacent to the redox-partner (POR) binding site (29–33). In humans, mutations in arginine residues 347 and 358 neutralize single positive charges in the redox-partner binding site and selectively impair C_{17-20} lyase activity (32), indicating that the region of P450c17 bordering the J-helix (Fig. 1) is important for redox-partner interaction.

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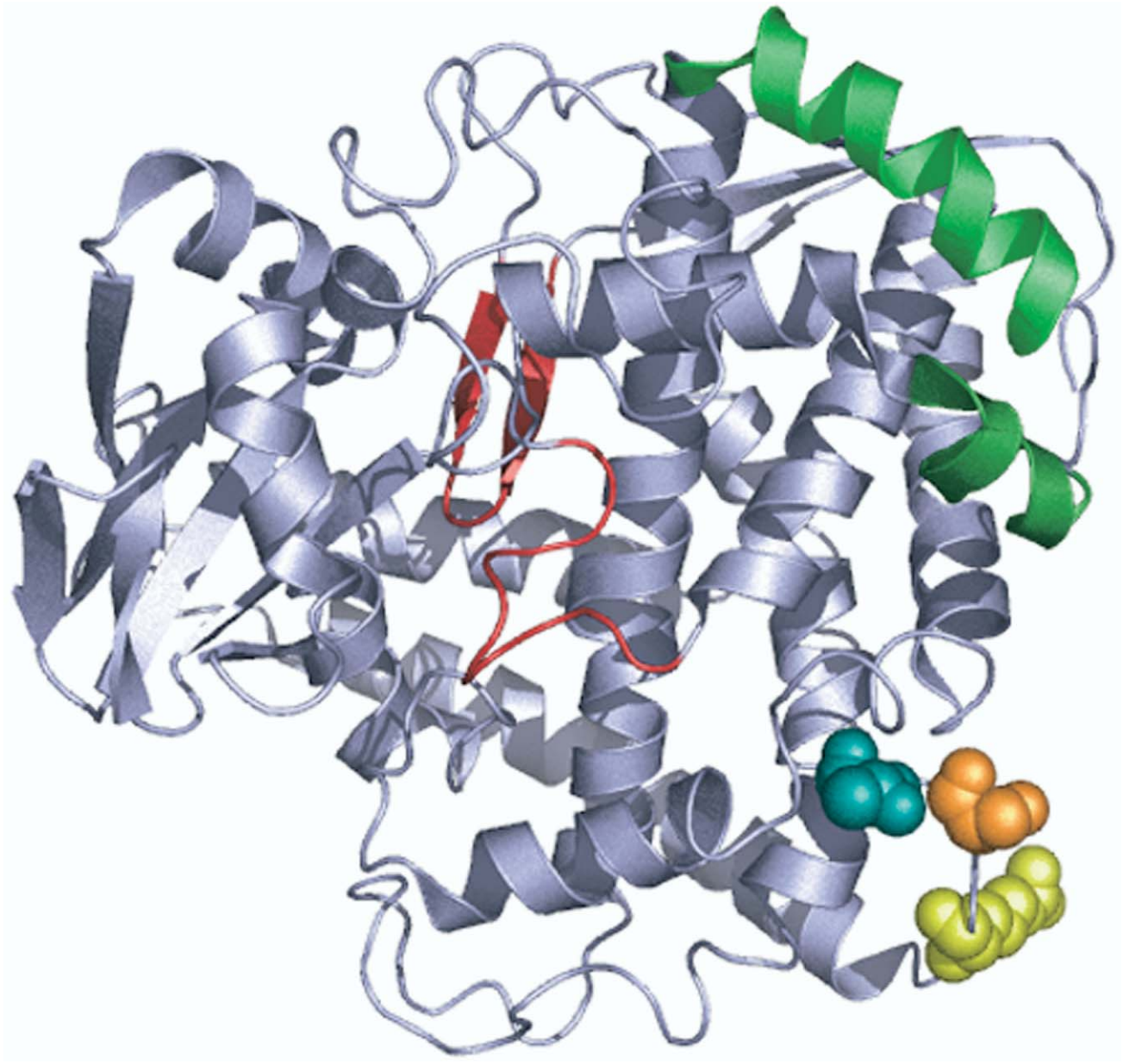
Adrenarche, an event contemporaneous with puberty but independent of gonadotropins and gonadal sex steroids (34–36), is a biochemical process that is confined to the human adrenal glands. It is associated with >100-fold rise in the concentrations of DHEA (37), without contemporaneous alterations in either glucocorticoid or mineralocorticoid production. Thus, adrenarche appears to occur by a selective up-regulation of the lyase function of the human P450c17, without concomitant change in its hydroxylase activity. Adrenarche occurs exclusively in human beings and other old-world primates (e.g., chimpanzees) (38, 39), and sequence analysis of the P450c17's reveals virtual identity between humans and chimps, between rhesus and baboons, and ~95% identity between the human–chimp and the rhesus–baboon families. Rhesus and baboon differ only at position 255, arginine in the baboon vs. histidine in the rhesus (which does not demonstrate adrenarche), suggesting that arginine 255 might play a role in the increase in P450c17

FIGURE 1

Ribbon diagram of P450c17. The redox-partner–binding region is predicted to be on the right face of the protein. *Dark green,* J′ helix; *light green,* J helix; *red,* substrate-binding pocket; *yellow,* arginine 255, a residue predicted to play a role in adrenarche; *orange,* serine 258; *cyan,* threonine 260.



Soulter. Site-directed mutagenesis of P450c17. Fertil Steril 2006.

activity observed at adrenarche (34). However, detailed kinetic analysis revealed that the substitution of histidine for arginine at position 255 caused no derangements in either its 17α-hydroxylase or C_{17-20} lyase activities. Conversely, substitution with alanine markedly diminished the hydroxylase function, indicating that the relative positive charge at position 255 may be required for redox-partner binding to promote hydroxylation.

In the ovary, the precise regulation of the onset and duration of androgen biosynthesis is crucial in maintaining follicular development and viability (40, 41). Inadequate androgen levels during preovulatory follicular development can result in reproductive failure, and the premature production of androgens in developing follicles can lead to follicular arrest and/or atresia (41, 42). Sustained elevated androgen production by the ovarian theca cells is a key etiologic feature of the polycystic ovarian syndrome (PCOS) (43, 44). The most consistent feature of PCOS is hyperandrogenism, which is of both ovarian and adrenal origin (45–49). The adrenal hyperandrogenism of PCOS resembles an exaggerated form of adrenarche, and girls with pronounced adrenarche and precocious pubarche are at risk for developing functional ovarian hyperandrogenism and PCOS (11, 50–52). A better understanding of the mechanisms that regulate the two constituent functions toward increased androgen biosynthesis might provide critical information regarding follicular growth, ovarian hyperandrogenism, and PCOS.

Because posttranslational phosphorylation mechanisms appear to alter the net activity of P450c17 in favor of increased androgen biosynthesis, we hypothesized that the phosphorylation of the serine and/or threonine residues proximal to arginine 255 might serve as a physiological regulator of P450c17 hydroxylase activity. Thus, the primary objective of this study was to evaluate the role of serine 258 and threonine 260 in the regulation of the activity of human P450c17. Our secondary objective was to develop a method to isolate the human P450c17 protein expressed in cells for further analysis of posttranslational modification of P450c17 and their effects in the catalytic activities.

MATERIALS AND METHODS

Materials

All primers were purchased from Qiagen Operon. Restriction endonucleases and COS cell growth media were purchased from GIBCO-BRL (Grand Island, NY). TLC Plates were purchased from Whatman (Clifton, NJ). All other reagents and supplies were obtained from standard sources. Institutional review board approval was not required because the study did not involve any human subjects.

Construction of FLAG-Tagged P450c17 Chromosomal DNA

Polymerase chain reaction (PCR) was used to create the P450c17 expression plasmids containing the eight-amino-acid flag epitope on either the C- or N-terminus of the protein. A full-length CYP17 chromosomal DNA (cDNA) was used as a template (6).

The following primers were used for the construction of the C-terminal FLAG-tagged P450c17: [1] sense primer: 5′-ATAGCTAGC **ATG** GACTACAAGGAT GACGACGATAAG TGGGAG CTCGTGGCT CTCTTG-3′; (the FLAG-coding nucleotides are underlined and the start codon is shown in bold) and [2] anti-sense primer: 5′-ATAGCGGCCGC **TTA** GGTGCTA CCCTCAGCCTG-3′ (the stop codon is shown in bold).

The primers used for the construction of the N-terminal FLAG-tagged P450c17 are as follows: [1] sense primer: 5′-ATAGCGGCCGC **TTA** CTTATCGTCGTCATCCTT GTAGTC GG TGCTACCCTCAGCCTG-3′ (the flag coding nucleotides are underlined and the stop codon is shown in bold) and [2] anti-sense primer: 5′-ATAGCTAGC**ATG** TGGGAGCTCGTGGCTCTC TTG-3′ (the start codon is shown in bold).

After PCR amplification, the cDNAs first were cloned into pCR 2.1-Topo (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The modified CYP17 cDNAs were subcloned into recombinant adenovirus using the BD Adeno-X expression system (BD Biosciences Clontech, Palo Alto, CA). The expression constructs were sequenced to verify that the epitope tag was inserted in frame and in the intended orientation and to ensure that no mutations were introduced into the CYP17 coding sequence. After packaging, purification, and titering, the recombinant adenoviruses were used to infect COS-7 cells to test the function of the tagged P450c17.

Site-Directed Mutagenesis of C-Terminus Flag-Tagged P450c17 cDNA

Full-length human C-terminal FLAG-tagged CYP17 cDNA, previously subcloned into pShuttle (BD Biosciences Clontech), was linearized with *Sal*I and subjected to PCR-based site-directed mutagenesis by using the Advantage High-Fidelity PCR kit (Clontech Laboratories, Inc.) according to the manufacturer's recommendations, by using the following primers:

[1] Construct S258A sense primer: 5′-GG AGT GAC **GCT** ATC ACC AAC-3′ and anti-sense primer: 5′-GTT GGT GAT **AGC** GTC ACT CC-3′ (codon 258 is in bold, the mutated base is underlined, the construct contains the point mutation GCT coding for alanine), [2] construct S258D sense primer: 5′-GG AGT GAC **GAT** ATC ACC AAC-3′ and antisense primer: 5′-GTT GGT GAT **ATC** GTC ACT CC-3′ (codon 258 is in bold, the mutated base is underlined, and the construct contains the point mutation GAT coding for aspartic acid), [3] construct T260A sense primer: 5′-GAC TCT ATC **GCC** AAC ATG CTG-3′ and anti-sense primer: 5′-CAG CAT GTT **GGC** GAT AGA GTC-3′ (codon 260 is in bold, the mutated base is underlined, the construct contains the point mutation GCT coding for ala-

nine), and [4] construct T260D sense primer: 5′-GAC TCT ATC **GAC** AAC ATG CTG-3′ and anti-sense primer: 5′-CAG CAT GTT **GTC** GAT AGA GTC-3′ (codon 260 is in bold, the mutated base is underlined, and the construct contains the point mutation GCT coding for aspartic acid).

Codons 258 (serine) and 260 (threonine) each were mutated to alanine (S258A, T260A) to prohibit phosphorylation and to aspartic acid (S258D, T260D) to generate pseudo-phosphorylated sites, respectively. The mutations were introduced into the CYP17 cDNA by sequential PCR using overlapping mutagenic oligonucleotides (53). The first step in the procedure involved synthesis of single-stranded DNA by using the overlapping but divergent internal mutagenic primers. Subsequently, the 5′ and 3′ external primers were added to amplify the full-length coding region, and the resulting PCR products were used as templates for the final amplification of the full-length cDNA. The final PCR products were purified on 1% agarose gels, isolated with the QIA-Quick gel extraction kit (Qiagen, Valencia, CA), and ligated into pCR 2.1-Topo according to the manufacturer's instructions.

The mutant cDNAs then were subcloned into recombinant adenovirus by using the BD Adeno-X expression system (BD Biosciences Clontech, Palo Alto, CA). The sequence and orientation of all mutated regions generated by PCR were verified by double-stranded sequencing to ensure that only the desired amino acid substitutions were incorporated.

Culture of COS-7 Cells, Transfection, and Assays

COS-7 cells were seeded (60,000 cells per well) in 24-well tissue culture plates containing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin and were grown for 24 hours until they were 60%–80% confluent. The medium was aspirated, and 200 μL of medium containing the appropriate multiplicity of infection (MOI) of recombinant adenovirus was gently overlaid on the cell monolayer and dispersed evenly with all vectors infected in triplicate wells. After 2 hours, another 800 μL of growth medium was added, and the cells were cultured for an additional 24 hours. The cells then were transferred to serum-free medium and cultured for an additional 24 hours before they were stimulated and incubated with substrate for either hydroxylase or lyase assays.

17α-Hydroxylase and $C_{17–20}$ Lyase Assays

17α-Hydroxylase and $C_{17–20}$ lyase activities were measured by a modification of methods described elsewhere (10, 54). To measure 17-hydroxylase activity, COS-7 cells were incubated in DMEM–Ham's F-12 (1:1) containing 0.5 mg/mL of bovine serum albumin, 1 μM unlabeled P, and 0.2 μCi [1,2,6,7-^{3}H]P (NEN Life Science Products, Boston, MA; 114 Ci/mmol). To measure $C_{17–20}$ lyase activity, COS-7 cells were incubated with [1,2,6,7-^{3}H]17α-hydroxypregnenolone (50 Ci/mM; American Radiolabeled Chemicals, Inc., St. Louis, MO). After 3 hours of incubation at 37°C in a 5% CO_2–5% O_2–90% N_2 humidified atmosphere, the medium was harvested and extracted twice with diethyl ether, and the pooled extracts were dried under a stream of air. The extracted steroids were dissolved in ethyl acetate–isooctane (1:1 by volume) and were assayed by thin-layer chromatography on silica gel plates (Whatman) by using a 3:1 chloroform–ethylacetate solvent system.

Autoradiography was performed by exposing TLC plates saturated with EN^{3}HANCE spray (NEN Life Science Products) to autoradiographic film. After developing the films, the areas on the plate corresponding to the labeled P substrate and 17α-hydroxyprogesterone product were cut from the TLC plate and counted in a scintillation counter. Similarly, for the lyase assay, after developing the films, the areas on the plate corresponding to the labeled 17α-hydroxypregnenolone substrate and DHEA product were cut from the plate and quantitated in a scintillation counter. The identity of the spots was confirmed by comparing their migration with authentic [^{3}H]P and [^{3}H]17α-hydroxyprogesterone standards for the hydroxylase assay and with authentic [^{3}H]17α-hydroxypregnenolone and [^{3}H]-DHEA for the lyase assay.

Molecular Modeling

The model of CYP17 was obtained from the Protein Databank (55). Hydrogen atoms were incorporated as necessary, by using the BIOPOLYMER module of the Insight II package (Accelrys, Inc., San Diego, CA). The starting structure of mutants was obtained by mutating each of the four residues from the initial structure by using Modeller8, version 1 (56–59). Structures were refined by using the AMBER 7 (56) simulation package and the ff99 forcefield. The average refined structures were calculated by using the PTRAJ module of the AMBER package. Structural superpositions of mutants were performed by using the combinatorial extension method (60).

Statistical Analysis

Treatments were conducted in triplicate, and each experiment was repeated a minimum of three times. Mean values from independent experiments were statistically examined by using one-way analysis of variance. Values were determined to be significant when $P \leq .05$.

RESULTS

Enzymatic Activities of FLAG-Tagged P450c17 cDNA

To study the interaction of human P450c17 with electron donors and the mechanism by which phosphorylation and/or the introduction of mutations affects enzymatic activity, it is necessary to be able to isolate the P450c17 protein. Prior attempts to raise antisera to P450c17 suitable for immuno-

precipitation have proven largely unsuccessful. Therefore, in the present study, we employed an alternative strategy to improve our ability to isolate P450c17 by adding an epitope tag to the P450c17 termini.

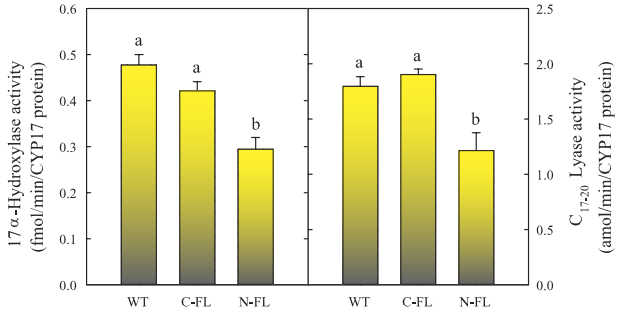
Recombinant adenoviruses expressing P450c17 with the eight-amino-acid FLAG tag on either the C- or N-terminus of the protein were generated. The expression constructs were sequenced to prove that the epitope tag was inserted in frame and in the intended orientation and to ensure that no mutations were introduced into the P450c17 coding sequence. COS-7 cells were infected with virus containing the cDNAs for wild-type (WT) P450c17, the C-terminus FLAG-tagged P450c17, and the N-terminus FLAG-tagged P450c17. As shown elsewhere, uninfected COS cells possess neither endogenous 17α-hydroxylase nor C_{17-20} lyase activities (10). COS-7 cells expressing WT P450c17 exhibited robust 17α-hydroxylase and C_{17-20} lyase activities, rapidly metabolizing progesterone (P) to 17α-hydroxyprogesterone and metabolizing 17α-hydroxypregnenolone to DHEA.

As shown in Figure 2, addition of a FLAG tag to the carboxy terminus had no effect on either 17α-hydroxylase or C_{17-20} lyase activities. Conversely, introduction of an N-terminal FLAG tag caused a 30%–40% decrease in both the 17α-hydroxylase (0.48 $\pm$ 0.03 vs. 0.29 $\pm$ 0.03 fmol/min) and C_{17-20} lyase (1.80 $\pm$ 0.09 vs. 1.2 $\pm$ 0.16 fmol/min) activities of human P450c17.

Cyclic adenosine 3′:5′ monophosphate (cAMP) has been shown to increase phosphorylation of P450c17 and increase C_{17-20} lyase activity (27, 61). To assess the effect of the FLAG tags on cAMP-induced C_{17-20} lyase activity, infected COS cells were treated with 8Br-cAMP, and the lyase–hydroxylase ratio was measured. Neither tag interfered with the cAMP-stimulated increase (1.7-fold) in lyase–hydroxylase ratio. Because the C-terminal FLAG-tagged protein functioned indistinguishably from the WT P450c17, we selected the C-terminal flag-tagged P450c17 for further characterization.

FIGURE 2

Expression of FLAG-tagged CYP17 in COS-7 cells. COS-7 cells were infected with adenovirus containing natural sequence CYP17 (WT), C-terminal FLAG-tagged CYP17 (C-FL), or N-terminal FLAG-tagged CYP17 (N-FL) and cultured for 2 days. The cells were incubated with radiolabeled substrates for 4 hours. The products were extracted from the medium, separated on TLC plates and quantitated by scintillation counting. Data are the mean $\pm$ SEM. Bars with different letters are significantly different, $P<.05$.



Soulter. Site-directed mutagenesis of P450c17. Fertil Steril 2006.

Characterization of Potential Phosphorylation Sites Adjacent to Arginine 255

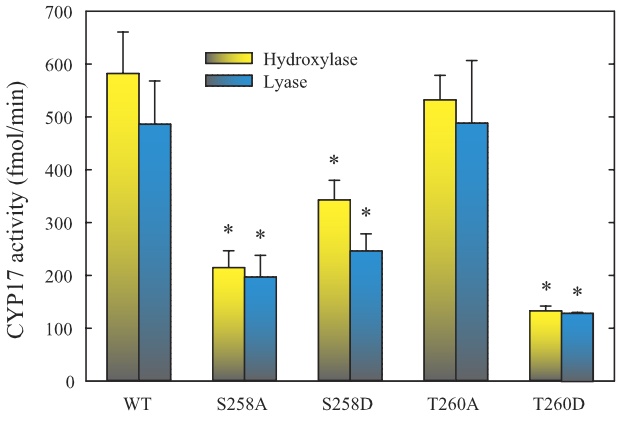
We employed the published computer-generated model of human P450c17 (30) to identify the potential phosphorylation sites, nearest to the arginine 255 (a residue that had previously been proposed to play a role in the increase in androgen production during adrenarche). The serine and/or threonine residues closest to arginine 255 were serine 258 and threonine 260. Taking advantage of the model structure, the positions of these two residues were analyzed and shown to be accessible for possible phosphorylation on the surface of the protein containing the J-helix that is thought to contain the redox-partner binding region (Fig. 1). Further analysis of the primary structure by using NetPHOS (62) and PROSITE (63) revealed that serine 258 falls within motifs predicted to be recognition sites for type II calcium–calmodulin-dependent protein kinase (recognition sequence, R-X-X-S/T-X), p70s6K (recognition sequence, K/R-X-R-X-X-S/T-X) and/or protein kinase A (recognition sequence, R-X_{1-2}-S/T-X). Similarly, threonine 260 falls within a predicted creatine kinase I recognition sequence (recognition sequence, Sp/Tp-X_{2-3}-S/T-X). Taken together, these observations suggested that both serine 258 and threonine 260 might fulfill criteria as potential phosphorylation sites capable of influencing redox-partner binding to P450c17.

Effect of Serine 258 and Threonine 260 Mutations on P450c17 Activities

Given the proximity of serine 258 and threonine 260 to Arginine 255, a residue that appears to have an important role in modulating 17α-hydroxylase/C_{17-20} lyase activity (34), and the results of the computer-modeling analysis, we tested the hypothesis that one or both of these residues might be involved in regulating the interaction of P450c17 with P450 oxidoreductase. Serine 258 and threonine 260 were each mutated to either alanine (S258A and T260A) or aspartic acid (S258D and T260D) in the human C-terminal FLAG-tagged P450c17 using site-directed mutagenesis. The mutant P450c17 constructs were packaged into adenovirus, and COS-7 cells then were infected with viruses containing the WT, S258A, S258D, T260A, or T260D P450c17 mutants. After culture for 48 hours to allow for expression of P450c17, the 17α-hydroxylase and C_{17-20} lyase activities were assayed in the presence of 8Br-cAMP to stimulate maximal phosphorylation of P450c17 and C_{17-20} lyase activity.

FIGURE 3

Effect of S258 and Thr260 mutations on P450c17 activity. COS-7 cells were infected with adenovirus containing either natural sequence CYP17 (WT), S258A, S258D, T260A, or T260D P450c17 and cultured for 2 days. The cells were treated with 8Br-cAMP in the presence of radiolabeled substrates for 4 hours. The steroid products were extracted from the medium, separated on TLC plates, and quantitated by scintillation counting. Data are mean ± SEM. Bars with asterisks are significantly different from WT, $P<.05$.



Soulter. Site-directed mutagenesis of P450c17. Fertil Steril 2006.

As shown in Figure 3, WT P450c17 exhibited robust 17α-hydroxylase and C_{17-20} lyase activities, as expected. The T260A mutant had equivalent 17α-hydroxylase activity (91.5%; 532 ± 47 fmol/min vs. 582 ± 79 fmol/min; *P*: not statistically significant) compared with the activity of the WT P450c17. In contrast, the S258A, S258D, and T260D mutants demonstrated considerably lower 17α-hydroxylase activity than the WT enzyme (215 ± 32 fmol/min, 343 ± 37 fmol/min, 133 ± 9 fmol/min vs. 582 ± 79 fmol/min, respectively, $P < 0.05$). The effects of each mutation on C_{17-20} lyase activity were similar to those observed with hydroxylase: T260A P450c17 exhibited C_{17-20} lyase activity similar to that of WT, whereas T260D, S258A, and S258D exhibited considerably less (128 ± 2 fmol/min, 197 ± 41 fmol/min, 246 ± 33 fmol/min vs. 486 ± 82 fmol/min, respectively, $P < 0.05$) activity.

Effect of Serine 258 and Threonine 260 Mutations on P450c17 Structure

There are two principal alternative explanations of the mutagenesis results. In one case, the mutations could disrupt the structure of the substrate-binding pocket, causing the enzyme to lose catalytic activity. Alternatively, the mutations could interfere with the interaction of P450c17 with P450 oxidoreductase and thereby inhibit electron transfer, resulting in diminished catalytic activity. To help interpret the results of the mutagenesis studies, molecular modeling was used to predict the structural changes in the P450c17 protein as a result of the mutations. Superpositions of the mutant models with the native structure reveal that the major alterations were on the surface of P450c17, immediately proximal to the mutations.

As shown in Figure 4, the mutations appear to disrupt what may be a binding surface spanning from arginine 255 to the J and J′ helices. However, the mutants did not appear to cause changes in the substrate-binding pocket itself, which is located on an alternate face of the P450c17 molecule.

DISCUSSION

Regulation of androgen biosynthesis by cytochrome P450c17 is paramount for follicular health and ovulation. However, the mechanisms promoting the inappropriate or excessive production of androgen are poorly understood. It therefore is important to investigate the mechanisms by which the enzymatic activities of human P450c17 are governed, with the goal of understanding the molecular basis for androgen-induced follicular atresia, ovarian hyperandrogenism, PCOS, and premature adrenarche.

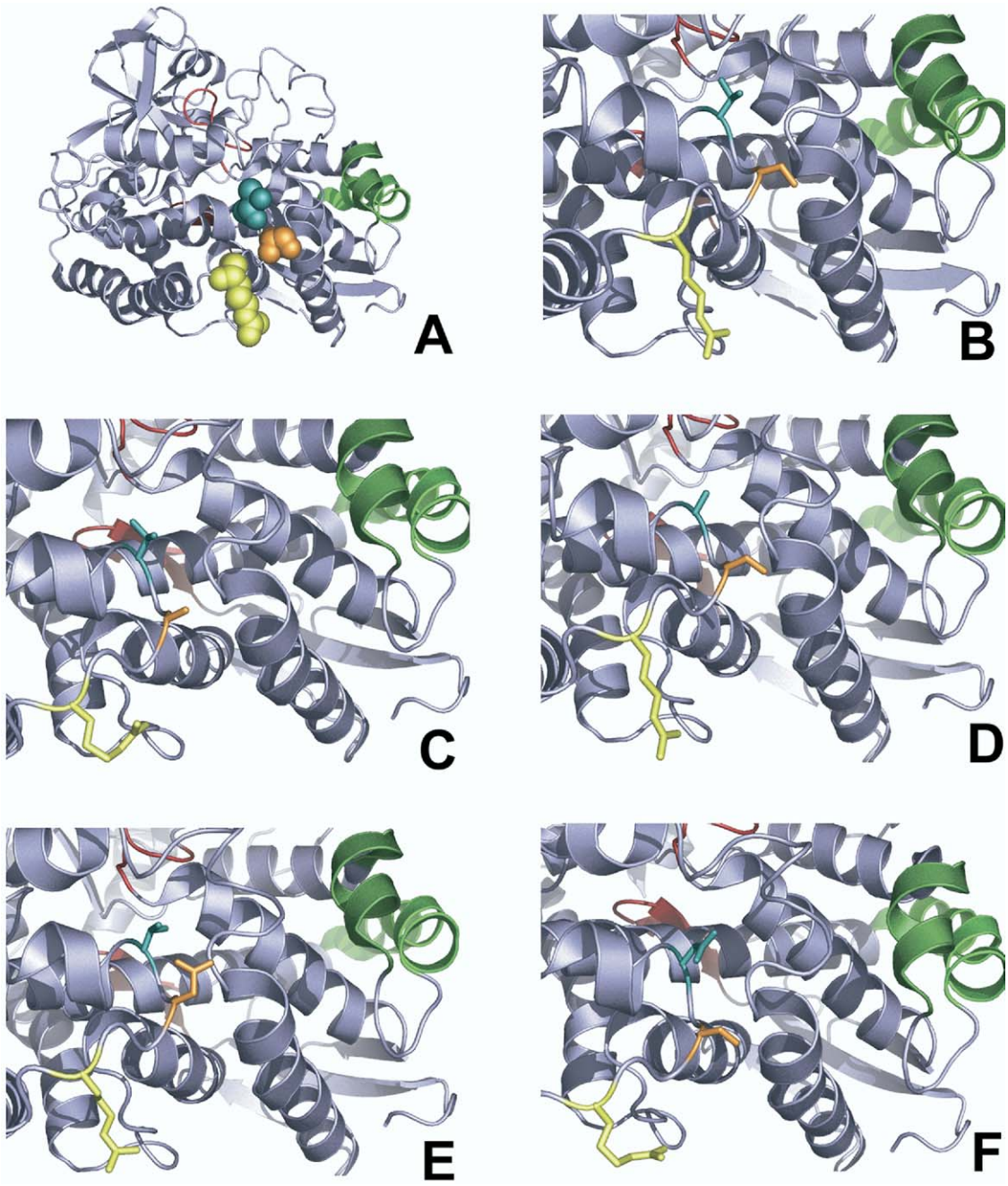
We initially sought to develop an epitope-tagged P450c17 to facilitate isolating the human protein expressed in cultured cells. The FLAG epitope was chosen because of its small size (eight amino acids) and the expectation that it would not interfere with enzyme activity. When compared with the WT enzyme, FLAG-tagged P450c17 with either end of the protein modified possessed both 17α-hydroxylase and C_{17-20} lyase activities. Uniquely, the C-terminal epitope tag had no effect on either activity, nor were discernible differences observed in phosphorylation-dependent augmentation of C_{17-20} lyase activity. This epitope-tagged P450c17 will be useful for the facile isolation of WT and mutant proteins expressed in cells for further analysis of posttranslational modifications of P450c17 and their effects on the catalytic activities.

On the basis of the observation that arginine 255 is a highly conserved residue that diverges in some of our closest primate relatives (e.g., rhesus) who do not demonstrate adrenarche, a maturational process resulting in increased C-19 steroid hormone production by the adrenal gland (34), we proposed that phosphorylation of one or more serine or threonine residues proximal to this region of the molecule might be involved in regulation of catalytic activity. The results of our sequence and three-dimensional analyses of protein structure supported this hypothesis. There are both serine and threonine residues adjacent to arginine 255 in three-dimensional space that are accessible for potential phosphorylation and that lie within the consensus recognition sequences for known serine–threonine kinases.

The technique of site-directed mutagenesis was used to gain further insight into the potential role of serine 258 and threonine 260 with respect to P450c17 activities. Mutation of serine 258 to either alanine or aspartic acid

FIGURE 4

Predicted structural changes induced by mutations to P450c17. Molecular modeling as described in the methods was used to depict predicted alterations in the structure of the P450c17 protein caused by mutations to serine 258 and threonine 260. (**A**) Ribbon diagram of P450c17 reoriented from Figure 1 to show the region containing the mutations more clearly. (**B–F**) Magnification of the region containing the mutations. (**B**) Native sequence P450c17. (**C**) S258A mutation. (**D**) T260A mutation. (**E**) S258D mutation. (**F**) T260D mutation. *Dark green,* J′ helix; *light green,* J helix; *red,* substrate-binding pocket; *yellow,* arginine 255; *orange,* serine 258; *cyan,* threonine 260.



Soulter. Site-directed mutagenesis of P450c17. Fertil Steril 2006.

caused marked decreases in both 17α-hydroxylase and C_{17-20} lyase activities, perhaps suggesting a critical role for serine 258 in the governance of P450c17 activity. In silico comparison of the three-dimensional structures of these mutant P450c17s with the WT protein indicates that there are no major alterations at the active site. It therefore is unlikely that the mutations of serine 258 caused conformational or structural defects in the protein that explain the loss of activity. Mutation of threonine 260 to alanine had no discernible effect on activity, indicating that phosphorylation of this residue is unlikely to play a significant role in the regulation of activity. Mutation of threonine 260 to aspartic acid caused a major loss of both activities. As with the serine 258 mutations, there were no significant alterations in the configuration of the protein structure or the active site as a result of mutations at threonine 260. Thus, it is unlikely that the mutations interfere with the ability of P450c17 to bind substrate. These data are most consistent with the interpretation that the region of P450c17 containing arginine 255, serine 258, and threonine 260 is important for interaction with OR. This interpretation is supported by the lack of structural change in the protein and active site and by the similar magnitude of the mutational effects on both 17α-hydroxylase and C_{17-20} lyase activities.

It is clear that this region is not involved in the selective regulation of C_{17-20} lyase activity. Selective regulation of C_{17-20} lyase activity is phosphorylation dependent (27). We employed mutation of serine 258 and threonine 260 to aspartic acid in an attempt to simulate a pseudo-phosphorylated residue at these positions. In both cases, there was a marked loss of both 17α-hydroxlase and C_{17-20} lyase activities. Although not conclusive, our data do not support a role for phosphorylation of either residue in redox-partner interactions.

In conclusion, our data extend our knowledge by demonstrating that serine 258 and threonine 260 are not involved in the selective regulation of the lyase activity; however, this region appears to be important for the interaction of P450c17 with its obligate redox partners, thereby affecting the catalytic activity of the enzyme. On the basis of our data and the information derived from naturally occurring mutations, it appears that there are at least two distinct regions of the face of P450c17 containing the J-helix that are important for redox-partner interactions. One involves the region including arginine 255, serine 258, and threonine 260, and the other involves the region adjacent to the J-helix. A serine at position 258 appears to be more critical for the preservation of activities than the threonine at position 260, given the lack of effect on activity as a result of the alanine substitution at 260. It is possible that the mutation to aspartic acid was not a sufficient facsimile of a phosphorylated serine to mimic its effect on the lyase activity; future experiments will substitute glutamic acid for the serines to be studied. How these regions affect the interactions of P450c17 with OR and/or cytochrome b_5 and their relation to the regulation of androgen production by the adrenal and gonads will require additional study.

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