Phosphorylation of Human Cytochrome P450c17 by p38α Selectively Increases 17,20 Lyase Activity and Androgen Biosynthesis*

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**Background:** Ser/Thr phosphorylation of P450c17 increases 17,20 lyase activity and androgenic capacity.

**Results:** Drug inhibition and siRNA knockdowns in adrenal cells implicate p38α, which phosphorylated bacterially expressed P450c17, doubling 17,20 lyase activity.

**Conclusion:** Phosphorylation of P450c17 by p38α provides a post-translational mechanism distinguishing glucocorticoid from sex steroid synthesis.

**Significance:** p38α pathways may participate in hyperandrogenic states and provide targets for glucocorticoid-sparing inhibition of androgen synthesis.

Cytochrome P450c17, a steroidalogen enzyme encoded by the CYP17A1 gene, catalyzes the steroid 17α-hydroxylation needed for glucocorticoid synthesis, which may or may not be followed by 17,20 lyase activity needed for sex steroid synthesis. Whether or not P450c17 catalyzes 17,20 lyase activity is determined by three post-translational mechanisms influencing availability of reducing equivalents donated by P450 oxidoreductase (POR). These are increased amounts of POR, the allosteric action of cytochrome b5 to promote POR-P450c17 interaction, and Ser/Thr phosphorylation of P450c17, which also appears to promote POR-P450c17 interaction. The kinase(s) that phosphorylates P450c17 is unknown. In a series of kinase inhibition experiments, the pyridinyl imidazole drugs SB202190 and SB203580 inhibited 17,20 lyase but not 17α-hydroxylase activity in human adrenocortical HCl-H295A cells, suggesting an action on p38α or p38β. Co-transfection of non-steroidalogen COS-1 cells with P450c17 and p38 expression vectors showed that p38α, but not p38β, conferred 17,20 lyase activity on P450c17. Antibserum to P450c17 co-immunoprecipitated P450c17 and both p38 isoforms; however, knockdown of p38α, but not knockdown of p38β, inhibited 17,20 lyase activity in NCI-H295A cells. Bacterially expressed human P450c17 was phosphorylated by p38α in vitro at a non-canonical site, conferring increased 17,20 lyase activity. This phosphorylation increased the maximum velocity, but not the Michaelis constant, of the 17,20 lyase reaction. p38α phosphorylates P450c17 in a fashion that confers increased 17,20 lyase activity, implying that the production of adrenal androgens (adrenarche) is a regulated event.

Three classes of steroid hormones are required for mammalian life: mineralocorticoids regulate renal sodium retention, thus regulating intravascular volume and blood pressure; glucocorticoids regulate carbohydrate metabolism and responses to stress; and sex steroids (androgens and estrogens) are required for reproduction of the species. In most mammals, mineralocorticoids are C21 (21-carbon) 17-deoxy steroids, glucocorticoids are C21 17-hydroxysteroids, and sex steroids are produced from C19 steroids. A single steroidalogen enzyme, cytochrome P450c17, encoded by the CYP17A1 gene determines which class of steroid is produced. P450c17 catalyzes both the 17α-hydroxylase activity needed to convert C21 17-deoxy steroids to their 17-hydroxy counterparts and the 17,20 lyase activity that cleaves the bond between carbon atoms 17 and 20 to convert C21 steroids to C19 steroids (for a review, see Ref. (1)). In the absence of P450c17, the human adrenal zona glomerulosa produces the mineralocorticoids deoxycorticosterone and aldosterone; in the adrenal zona fasciculata, P450c17 catalyzes 17α-hydroxylase activity but not 17,20 lyase activity to produce the glucocorticoid cortisol; and in the adrenal zona reticularis, testicular Leydig cells, and ovarian theca cells, P450c17 produces dehydroepiandrosterone (DHEA)2 and androstenedione, the principal C19 steroids that are converted to testosterone and estradiol (see Fig. 1A). P450c17 is an evolutionarily ancient enzyme essential for sex steroid synthesis in all vertebrates. Thus, P450c17 is the qualitative regulator of steroidogenesis, determining the class of steroid produced. The 17,20 lyase activity of P450c17 varies considerably among species: pig (2–4), trout (5), and frog (6) P450c17 catalyze 17,20 lyase activity with both 17-OH-pregnenolone (17-OH-Preg; a C21 steroid) and 17-OH-progesterone (a C19 steroid), bovine P450c17 also catalyzes 17,20 lyase activity with both substrates but with a preference for 17-OH-Preg (7); rat (8) and presumably mouse, which only differs by two residues), hamster (9),

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2 The abbreviations used are: DHEA, dehydroepiandrosterone; POR, P450 oxidoreductase; Preg, pregnenolone; POPS, polycystic ovary syndrome; ROCK1, Rho-associated, coiled coil-containing protein kinase 1; 8-Br-cAMP, 8-bromoadenosine 3’,5’-cyclic monophosphate; Ni-NTA, nickel-nitrilotriacetic acid; M3K, mitogen-activated protein kinase kinase; PP, protein phosphatase; M KK, mitogen-activated protein kinase kinase.
Phosphorylation of P450c17 by p38α

and guinea pig (10) P450c17 strongly prefer 17-OH-progesterone; and human P450c17 catalyzes 17,20 lyase activity almost exclusively with 17-OH-Preg (11–13).

The single human CYP17A1 gene (14) produces a single species of mRNA (15) and protein, which catalyzes both the hydroxylase and lyase activities on a single active site (16). Nevertheless, the 17,20 lyase activity of human adrenal P450c17 is developmentally regulated. Concentrations of cortisol, an index of 17α-hydroxylase activity, remain essentially constant as a function of age, whereas concentrations of DHEA and its sulfate rise 100-fold at adrenarche (17), which is an event in the primate adrenal that is approximately contemporaneous with but independent of puberty (18–20). Thus, the mechanisms separately regulating these two activities of P450c17 are of substantial enzymological interest.

As with other microsomal (type 2) cytochrome P450 enzymes, catalysis by P450c17 begins with transfer of two electrons from NADPH to the flavin adenine dinucleotide (FAD) moiety of the two-flavin protein P450 oxidoreductase (POR). Electron acceptance by the FAD moiety elicits a conformational change in POR, bringing the FAD close to the flavin mononucleotide (FMN) moiety, which then accepts the electrons from the FAD; the POR molecule then reverts to its original, more open conformation, permitting the FMN domain to dock by charge-charge interactions with the redox partner binding site of the P450 acceptor molecule (21–24). The electrons then flow to the heme iron of P450c17, which mediates catalysis of both the 17-hydroxylase and 17,20 lyase reactions apparently by a ferryl oxene mechanism (16) (Fig. 1B).

Because of its key role in the production of sex steroids, its potential role in hyperandrogenic disorders, and its potential as a therapeutic target for sex steroid-dependent cancers, the 17,20 lyase activity of human P450c17 has received increased attention (25, 26). Studies with pure recombinant proteins and with transfected cells indicate that 17,20 lyase activity is controlled by factors that influence the efficiency of electron transfer from P450 oxidoreductase to P450c17 (27, 28). Thus, 17,20 lyase activity can be augmented by three post-translational modifications: first, Mn2+ promotes the interaction of P450c17 and POR (34), indicating that both factors operate via the same mechanism, presumably optimizing interactions with POR. The polycystic ovary syndrome (PCOS) is a hyperandrogenic disorder affecting about 6% of women of reproductive age (41, 42). There are multiple forms of PCOS: some women appear to have an autosomal dominant disorder characterized by both hyperandrogenemia and insulin resistance (43–45). Because Ser/Thr phosphorylation of P450c17 will increase 17,20 lyase activity (34, 37–40). Either maximal Ser/Thr phosphorylation of P450c17 or maximal concentrations of b5 will maximize 17,20 lyase activity so that adding b5 to phosphorylated P450c17 elicits no further increase in lyase activity (34), indicating that both factors operate via the same mechanism, presumably optimizing interactions with POR.
the β chain of the insulin receptor (52, 53) or its substrate IRS-1 (54) appears to be involved. However, the Ser/Thr kinase(s) that phosphorylates P450c17 and/or the β chain of the insulin receptor has not been identified.

The phosphorylation of P450c17 in human NCI-H295 adrenocortical carcinoma cells appears to be inducible by cAMP (37) and can be reversed by protein phosphatase 2A (PP2A) but not by PP4 or PP6 (39). In an initial search for such a kinase, we performed microarray and inhibitor studies in NCI-H295A cells (55). Of the 518 kinases in the human “kinome,” 278 are Ser/Thr kinases (56), and microarrays identified only 145 of these Ser/Thr kinases in NCI-H295A cells. Several kinases that are implicated in insulin action were absent, including protein kinase A (PKA), mammalian target of rapamycin, phosphatidylinositol 3-kinase (PI3K), mitogen-activated kinase 3 (MAPK3)/extracellular signal-regulated kinase 1 (ERK1), MAPK1/ERK2, MAP2K1/mitogen-activated protein kinase kinase 1 (MEK1), and MAP2K2/MEK2. Thus, the pathways involving ERK1/2 and MEK1/2 that have been implicated in the insulin resistance of PCOS muscle cells (57, 58) cannot be excluded the PKA/PI3K/Akt and calcium/calmodulin/MEK pathways and suggested a role for Rho-associated, coiled coil-containing protein kinase 1 (ROCK1). However, although recombinant ROCK1 could phosphorylate P450c17 in vitro, this phosphorylation did not affect 17,20 lyase activity; hence ROCK1 may act upstream in a pathway leading to the unidentified kinase (55). Of the 145 Ser/Thr kinases found in NCI-H295A cells, only six were induced more than 2-fold by 8-Br-cAMP, and knockdown of each of these by RNA interference had no effect on 17,20 lyase activity (55). One of the cAMP-inducible kinases was MAPK13, also known as p38α, which is a major okadaic acid-responsive MAPK and can inactivate ERK1/2 activity directly (59). Therefore, we considered the potential roles of other p38 isoforms. This family of Ser/Thr kinases consists of p38α (MAPK14), p38β (MAPK11), p38δ (MAPK13), and p38γ (MAPK12); p38α and p38β are expressed ubiquitously, whereas p38δ is confined to muscle, and p38γ is found in lung, kidney, gut, and some endocrine tissues (60). Both p38α and p38β have been implicated in regulating steroidogenesis (61–63).

**EXPERIMENTAL PROCEDURES**

**Materials**—Activated recombinant human p38α and recombinant cytochrome b5 were from Invitrogen. Pyridinyl imidazole drugs SB202190 and SB203580 were from EMD Millipore (Billerica, MA). Our anti-P450c17 antiserum has been described (11), and anti-FLAG antibody was from Sigma-Aldrich. p38α and p38β expression vectors were kindly provided by Dr. Jiahuai Han (The Scripps Research Institute) (64).

**Cell Cultures and Transfections**—NCI-H295A cells are an adherent subline (65) of human adrenocortical carcinoma NCI-H295 cells (66) that express all adrenal steroidogenic enzymes in a physiologically appropriate, hormonally responsive fashion (67). Cells were cultured in plastic dishes at 37 °C and 5% CO2 in RPMI 1640 medium supplemented with 2% fetal calf serum (FCS), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 50 μg/ml gentamycin. Cells were transfected using Effectene (Qiagen, Valencia, CA) according to the manufacturer’s protocol.

**Bacterial Expression of P450c17 and POR**—The pCWH17-mod(His)4 expression plasmid containing the cDNA for human P450c17 with N-terminal modifications that facilitate bacterial expression (68) and modified to contain three glycines followed by six histidines (C-terminal G6H6 extension) (69) or a vector expressing human POR carrying a C-terminal G6H6 extension and lacking 27 N-terminal residues (70) was transformed into Escherichia coli strain JM109. A single colony of E. coli strain JM109 transformed with pCWH17mod(G6H6) was first grown to saturation in 10 ml of Luria-Bertani medium containing 100 μg/ml ampicillin at 37 °C with shaking at 220 rpm and then added to 1 liter of terrific broth containing 100 μg/ml carbenicillin, 40 μg/ml FeCl3, 4 μM ZnCl2, 2 μM CoCl2, 2 μM Na2MoO4, 2 μM CaCl2, 2 μM CuCl2, 2 μM H3BO3, 1 mM thiamine and grown at 37 °C with shaking at 220 rpm to an A600 of 0.2; δ-aminolevulinic acid was then added to 0.5 mM. The culture temperature was lowered to 28 °C, and at an A600 of 0.4, isopropyl 1-thio-β-D-galactopyranoside was added to 0.5 mM, and the culture was shaken at 120–150 rpm at 28 °C for 2 days. The culture was iced to 4 °C, and the bacteria were harvested at 5,000 × g for 10 min and resuspended in 20 ml of 0.1 M Tris acetate, pH 7.8, 0.5 mM EDTA, 0.5 mM sucrose (TES buffer), and lysosome was added to 0.2 mg/ml for ≥2 h. Spheroplasts were collected at 12,000 × g for 10 min and homogenized in 50 mM potassium phosphate, pH 7.4, 10 mM MgCl2, 0.1 mM EDTA, 20% glycerol, 1 mM DTT, 40 μM progesterone, 0.2 mM PMSF, 1 μg/ml DNase 1 (buffer A). Homogenized spheroplasts were sonicated at 4 °C with eight to nine cycles of 25 s on, 30 s off using a 550 Sonic Dismembranator (Fisher Scientific) at 30% power. The lysate was cleared by centrifugation at 12,000 × g for 10 min, and the supernatant was centrifuged at 265,000 × g for 45 min to pellet the membranes. The membrane-associated P450c17 in the pellet was solubilized in 50 mM potassium phosphate, pH 7.4, 20% glycerol, 0.5% Triton X-114, 0.2% sodium cholate, 10 mM imidazole, 40 μM progesterone, 0.1 mM PMSF (buffer B) and then cleared by centrifugation at 27,000 × g for 25 min. The supernatant (20 ml) was loaded slowly onto a Ni-NTA-agarose column (1.6 × 2.5 cm), which had been pre-equilibrated with 25 mM potassium phosphate, pH 7.4, 10% glycerol, 0.1% Triton X-100, 0.1% sodium cholate, 10 mM imidazole, 40 μM progesterone, 0.1 mM PMSF (buffer C). The column was washed with buffer C, then with buffer D (buffer C plus 300 mM NaCl), and finally with 25 mM potassium phosphate, pH 7.4, 10% glycerol, 0.1% Triton X-100, 0.1% sodium cholate, 50 mM imidazole, 40 μM progesterone, 0.1 mM PMSF (buffer E). The P450c17 was eluted with 25 mM potassium phosphate, pH 7.4, 10% glycerol, 0.2% sodium cholate, 300 mM imidazole, 0.1 mM PMSF (buffer F) and desalted into 25 mM potassium phosphate, pH 7.4, 10% glycerol, 0.2 mM DTT, 0.1% sodium cholate (buffer G) by passage through a Sephadex G-25 column (1.6 × 10 cm) or by dialysis against Buffer G. All purification steps were performed at ~0–4 °C. Protein concentration was determined by the Bradford method, and the purity was examined by SDS-PAGE and Coomassie Brilliant Blue staining. P450 content was determined using a molar extinction
Phosphorylation of P450c17 by p38α

TABLE 1

| Type of assay                  | Sequences (5’ to 3’)                                                                 |
|-------------------------------|--------------------------------------------------------------------------------------|
| shRNA                         |                                                                                      |
| MAPK14 (nt 942–960 in NCBI Reference Sequence NM_001315.2) | GTAACTCTAGCCATAGTGAGGA                                                             |
| MAPK11 (nt 815–833 in NCBI Reference Sequence NM_002751.5) | GAACACGCCCGAGACATATA                                                            |
| Scrambled                     | ACATTGAAGCGAAGAATA                                                                 |

| Site-directed mutagenesis     |                                                                                      |
|-------------------------------|--------------------------------------------------------------------------------------|
| T341A                         | GGGCCACGCTCATCGAAGCTAT                                                             |
| T341D                         | GTGGGTTTCAAGGGCGACCCAACTATCAGTGAGC                                                 |
| T341E                         | GTGGGTTTCAGCCGCGACCCAACTATCAGTGAGC                                                 |
| S427A                         | GGACCACGCTCATCGAAGCTAT                                                             |
| S427D                         | GGACCACGCTCATCGAAGCTAT                                                             |
| S427E                         | GGACCACGCTCATCGAAGCTAT                                                             |

A difference of 91 cm⁻¹ mm⁻¹ between 450 and 490 nm (71). The base-line spectrum from 400 to 500 nm of 5 mg of protein was measured after the addition of 20 mg of sodium dithionite, and the reduced CO spectrum was measured after the sample was bubbled gently with carbon monoxide for 1 min.  

Mutagenesis and Expression—The P450c17 vector with the C-terminal G₁₃H₁₃ extension (69) was modified by site-directed mutagenesis as described (55) to express P450c17 carrying the mutations T341A, S427A, and T341A/S427A. Mutagenic oligonucleotide sequences are given in Table 1.  

Phosphorylation and Dephosphorylation of Human Wild-type and Mutant P450c17—Bacterially expressed wild-type human P450c17 and the mutants T341A, S427A, and T341A/S427A were incubated with catalytically active recombinant human P450c17 and the mutants T341A, S427A, and T341A/S427A. Mutagenic oligonucleotide sequences are given in Table 1.  

Preparation of Short Hairpin RNA (shRNA) Vectors and Knockdown Procedures—We constructed shRNA expression vectors to express short hairpin RNA constructs against p38α, p38β, and a control scrambled sequence in Lentilox PLL 3.7 (72). The constructs were co-transfected with packaging vectors into HEK-293T cells by Effectene (Qiagen) for 2 days. The supernatants containing lentiviral particles were filtered on 0.45-μm polyvinylidene difluoride membranes, used to transduce human adrenal NCI-H295A cells for 2 days, and assayed for 17-hydroxylase and 17,20 lyase activities.  

Assays of Steroidogenic Activities—[14C]Progesterone (114.4 Ci/mmol) and [3H]pregnenolone (22.9 Ci/mmol) were purchased from PerkinElmer Life Sciences. 17-OH-[3H]Preg was prepared from [3H]pregnenolone in collaboration with Professor Richard J. Auchus (University of Michigan, Ann Arbor, MI) as described (73) using bacterially expressed human P450c17 and POR prepared as described above. The [3H]Preg (~60 μCi) was dried under N₂, dissolved in 10 μl of EtOH, and combined with 0.4 ml of 50 mM potassium phosphate buffer, pH 7.4 supplemented with 6 mM potassium acetate, 10 mM MgCl₂, 1 mM reduced glutathione, 20% glycerol and also containing 1.8 μg of purified P450c17 (80 nm), 10 μg of purified human POR (320 nm), 30 μM dilaurylphosphatidylcholine, and 1 mM NADPH. The mixture was incubated for 40 min at 37 °C, and the steroid products were extracted with dichloromethane and dried under N₂. The extract was dissolved in 25 μl of dichloromethane and loaded onto a 2-ml column of silica gel (Dynamic Adsorbents, Norcross, GA) previously washed with hexane, and the steroids were eluted with a stepwise gradient of ethyl acetate (10–40%) in hexane. The eluate was collected in 2-ml fractions and assayed by HPLC, and the fractions containing 17-OH-Preg were pooled and concentrated. The resulting product was assayed by thin layer chromatography (TLC) in a 3:1 chloroform:methyl acetate solvent system as described (74); >98% of the radioactivity was in 17-OH-Preg.  

Enzyme Kinetics—Purified bacterially expressed wild-type or mutant P450c17 (10 pmol) with or without phosphorylation by p38α was assayed for 17,20 lyase activity with 20 pmol of POR in 100 mM potassium phosphate, 6 mM potassium acetate, 20 μg of 1,2-dioleoyl-sn-glycerol-3-phosphocholine, 10 mM MgCl₂, 1 mM reduced glutathione, 20% glycerol, 3 units of glucose-6-phosphate dehydrogenase, 0.1 mM glucose 6-phosphate and incubated for 3 h at 37 °C with 17-OH-[3H]Preg in a total volume of 200 μl. Steroids were extracted, analyzed by TLC, and quantitated. Lineweaver-Burk plots were made in GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analyses were performed using two-tailed unpaired t tests, and significance was accepted for tests where the p value was <0.05. P450c17 kinetic parameters (Kₘ and Vₘₚₓ) were calculated as mean ± S.E. (three independent experiments, each performed in triplicate), whereas all other comparisons were calculated as mean ± S.D.  

RESULTS

p38α Increases 17,20 Lyase Activity in Adrenal Cells—Among the kinases that were induced by cAMP in NCI-H295A cells was MAPK13 (55), also known as p38δ, which is a major okadaic acid-responsive MAPK and can inactivate ERK1/2 activity directly (59). Therefore, we considered the potential roles of other p38 isoforms. This family of Ser/Thr kinases consists of p38α (MAPK14), p38β (MAPK11), p38δ (MAPK13), and p38γ (MAPK12); p38α and p38β are expressed ubiquitously, whereas p38δ is confined to muscle, and p38γ is found in lung, kidney, gut, and some endocrine tissues (60). Both p38α and p38β have previously been implicated in regulating steroido-
Phosphorylation of P450c17 by p38α

FIGURE 2. p38α increases the 17,20 lyase activity of P450c17. A, TLC of one inhibitor experiment. Serum-starved NCI-H295A cells were treated for 4 h with the indicated doses (μM) of SB202190 and SB203580 and incubated with 1 μM [14C]progesterone (prog) (1 h) or 1 μM 17-OH-[3H]Preg (17-Preg) (1 h), and extracted media were analyzed by TLC. Both drugs inhibit 17,20 lyase activity (DHEA production) at 1 μM, B, pooled phosphorimagery data of three experiments done as in A. Data are compared with untreated control. Error bars represent S.E.; *, p < 0.05; **, p < 0.001.

AUGUST 16, 2013 • VOLUME 288 • NUMBER 33
Phosphorylation of P450c17 by p38α

A. Both p38α and p38β interact with P450c17 in vivo. COS-1 cells were co-transfected with P450c17 and FLAG-tagged p38α or p38β, and probed with anti-FLAG (lanes 1–3). The signal in lanes 2 and 3 shows expression of p38. COS-1 cell lysate was immunoprecipitated with anti-P450c17. Probing with anti-FLAG detects IgG in lanes 4–6 and co-immunoprecipitated p38α in lanes 5 and p38β in lane 6. B. p38α phosphorylates P450c17 in vitro. Purified P450c17 was incubated with [γ-32P]ATP without (lane 1) or with (lane 2) activated recombinant human p38α, captured onto Ni-NTA beads, washed, eluted, and analyzed by SDS-PAGE and autoradiography. Arrow, phospho-P450c17.

C. 17,20 Lyase activity of P450c17 phosphorylated in vitro with p38α. Purified P450c17 was incubated without (duplicate lanes 1 and 2) or with (duplicate lanes 3 and 4) p38α and “cold” ATP and assayed for 17,20 lyase activity in the presence of NADPH, recombinant POR, and 17-OH-[3H]Preg. D. shRNA constructs specifically knock down p38α and p38β. Constructs directed against p38α, p38β, and a control scrambled sequence were transformed into NCI-H295A cells for 2 days followed by RT-PCR of p38α, p38β, and GAPDH RNA. Lanes 1–3, shRNA against p38α reduces p38α but not p38β; lanes 4–6, shRNA against p38β reduces p38β but not p38α; lanes 7–9, none of the shRNAs affects GAPDH.

Knockdown of p38 Inhibits 17,20 Lyase Activity in Adrenal Cells—To determine whether a p38 kinase participates in the physiologic phosphorylation of P450c17 in vivo, we examined the effect of knocking down the expression of p38 isoforms in NCI-H295A cells. Using a lentivirus system (72), we built shRNA constructs designed to yield small inhibiting RNAs (siRNAs) against p38α, p38β, and a control scrambled sequence; propagated the viruses in HEK-293T cells; and then used the virus to transform NCI-H295A cells (Fig. 3D). Assays of the hydroxylase and lyase in the transformed cells showed that 17,20 lyase activity was inhibited 1.7-fold by shRNA knockdown of p38α but not by knockdown of p38β or by the scrambled shRNA control (Fig. 3, E and F). Thus, p38α augments the 17,20 lyase activity of P450c17 in vitro as well as in vivo.

p38α Phosphorylates P450c17 at a Non-canonical Site—The consensus amino acid recognition sequence for p38α substrates is (Ser/Thr)-Pro (76) with 159 of 191 sites phosphorylated by p38α following this rule; this recognition sequence is typically assisted by docking motifs that may be dozens of residues upstream (77, 78). The Ser/Thr residue(s) of P450c17 phosphorylated by p38α that results in the increased 17,20 lyase activity has not yet been identified. Human P450c17 has 32 Ser residues and 25 Thr residues (15) of which only two, Thr-431 and Ser-427, are immediately followed by a proline. To test whether these consensus phosphorylation sites participate in the p38α-induced increase in the 17,20 lyase activity of P450c17, they were mutagenized to Ala both singly (T341A and S427A) and in combination (T341A/S427A), expressed, and purified to apparent homogeneity. Despite the mutation of the p38α consensus phosphorylation sites, the mutant proteins were phosphorylated in vitro by p38α to an equivalent extent as wild-type P450c17 (Fig. 4A). We then assayed the activities of these bacterial expressed mutants in comparison with the wild-type protein. All three mutants retained their p38α-induced acquisition of 17,20 lyase activity at a level that was indistinguishable from wild-type P450c17 (Fig. 4B). Thus, p38α appears to phosphorylate P450c17 at a non-canonical site(s).

Phosphorylation of P450c17 Selectively Augments the Velocity of 17,20 Lyase Activity—To understand how phosphorylation of P450c17 augments its 17,20 lyase activity, we assessed the kinetics of the 17,20 lyase reaction as catalyzed by either non-
Phosphorylated P450c17 or P450c17 phosphorylated in vitro by p38α. Bacterially expressed, purified P450c17 was phosphorylated with p38α/H9251 and used for kinetic assays of 17,20 lyase activity compared with P450c17 that was not phosphorylated. Phosphorylation had no measurable effect on the maximum velocity (V_{max}) or Michaelis constant (K_{m}) of the 17α-hydroxylase reaction. By contrast, whereas both forms of P450c17 had similar Michaelis constants for the 17,20 lyase reaction, the phosphorylated P450c17 had a significantly greater V_{max} (Fig. 4, C and D).

A change in K_{m} would suggest a conformational change that affects steroid binding or release; however, the only change was an increased V_{max}. All available information about the promotion of 17,20 lyase activity converges on increasing the efficiency of electron receipt. Thus, our data suggest that the p38α/H9251-mediated phosphorylation of P450c17 affects its interaction with P450 oxidoreductase and hence substrate turnover.

Phosphorylation of P450c17 by p38α is Reversed by PP2A—We have shown previously that P450c17 that has been phosphorylated by endogenous kinase(s) from human adrenal NCI-H295A cells can be dephosphorylated by PP2A in a fashion that ablates kinase-induced 17,20 lyase activity (39). To test whether PP2A exerts a similar effect on P450c17 phosphorylated by p38α, we incubated bacterially expressed human P450c17 with p38α and [γ-32P]ATP and then dephosphorylated the phospho-P450c17 with PP2A. PP2A eliminated all detectable phosphate incorporation and returned the 17,20 lyase activity to the level seen without p38α phosphorylation (Fig. 5).
Phosphorylation of P450c17 by p38α

FIGURE 5. Phosphorylation of recombinant P450c17 by p38α is reversed by PP2A. A, P450c17 was phosphorylated by p38α and (γ-32P)ATP (lane 1) followed by incubation with either 1 (lane 2) or 10 units (lane 3) of PP2A and analyzed by autoradiography. Arrow, phospho-P450c17. B, 17,20 lyase activity of P450c17 phosphorylation by p38α and dephosphorylation by PP2A, ori, origin.

Phosphorylation of P450c17 by p38α

The traditional questions concerning adrenarche have concerned its regulation, its intracellular mechanisms, and its role in human physiology. A number of searches for a hypothetical, specific adrenal androgen-stimulating hormone that would regulate C19 steroid synthesis by the adrenal zona reticularis, analogous to angiotensin II acting on the zona glomerulosa or ACTH acting on the zona fasciculata, have failed. The PCOS is a complex disorder of unknown cause(s) characterized by adrenal and ovarian hyperandrogenism, insulin resistance, and obesity, although many affected women have only some of these features (42). Adrenarche that begins earlier and that results in higher concentrations of C19 steroids frequently precedes PCOS, and many now regard premature exaggerated adrenarche as an early form of PCOS (83–85). Because of such connections between adrenal C19 steroid production and metabolic regulation, factors not specific to the adrenal including nutrition (86), insulin (87), insulin-like factors (50, 88, 89), leptin (38), and fibroblast growth factor (90) have been considered as potential triggers of adrenarche. However, the degree to which P450c17 is phosphorylated in different androgen-producing cell types is unknown, and all such “top down” approaches have yielded results of uncertain significance largely because a cellular model of adrenarche has not been developed.

It has been suggested that the increased adrenal and ovarian C19 steroid production and insulin resistance in PCOS are connected by an unidentified signal transduction pathway that ultimately increases the Ser/Thr phosphorylation of both P450c17 and either the insulin receptor or its substrate (37, 52). However, despite numerous studies (55, 57, 58), a relevant kinase has not been identified. We have now shown that P450c17 can be phosphorylated by p38α in a fashion that selectively augments its 17,20 lyase activity, thus providing a proof of principle for the regulation of 17,20 lyase activity by P450c17 phosphorylation. This discovery now permits a new “bottom up” approach to the study of adrenarche. Because p38α and other MAPKs are typically terminal components of a three-kinase cascade (91, 92), one can now apply similar approaches to identifying the kinase (presumably an MKK) that phosphorylates p38α in turn permitting identification of the initial MAP3K and ultimately the factor(s) that triggers that kinase. Fig. 6 outlines our current speculation about the nature of this pathway. Hormones (e.g. insulin, insulin-like growth factors, leptin, etc.), environmental agents, and dietary factors would activate a pathway that may involve ROCK1, which has previously been implicated in P450c17 phosphorylation (55). ROCK1 might act as an upstream scaffolding protein in a MAPK pathway (93) or might activate one of a large number of mitogen-activated protein kinase kinases (M3Ks), which in turn would activate a mitogen-activated protein kinase kinase, most likely MKK3, -4, or -6, which are known to activate p38α and which are found in NCI-H295A cells (54). As shown herein, p38α can then phosphorylate P450c17 in a fashion that confers 17,20 lyase activity. We have shown previously that P450c17 is specifically dephosphorylated by PP2A, which in turn may be inhibited by the phosphoprotein SET. Potential cross-talk with other second messenger pathways is not shown. Steps that have been established experimentally are shown with closed arrowheads, and hypothetical steps are shown with open arrowheads.

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