Signaling regulates activity of DHCR24, the final enzyme in cholesterol synthesis

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Abstract  The role of signaling in regulating cholesterol homeostasis is gradually becoming more widely recognized. Here, we explored how kinases and phosphorylation sites regulate the activity of the enzyme involved in the final step of cholesterol synthesis, 3β-hydroxysterol Δ24-reductase (DHCR24). Many factors are known to regulate DHCR24 transcriptionally, but little is known about its posttranslational regulation. We developed a system to specifically test human ectopic DHCR24 activity in a model cell-line (Chinese hamster ovary-7) using siRNA targeted only to hamster DHCR24, thus ensuring that all activity could be attributed to the human enzyme. We determined the effect of known phosphorylation sites and found that mutating certain residues (T110, Y299, and Y507) inhibited DHCR24 activity. In addition, inhibitors of protein kinase C ablated DHCR24 activity, although not through a known phosphorylation site. Our data indicate a novel mechanism whereby DHCR24 activity is regulated by signaling.

Supplementary key words  3β-hydroxysterol Δ24-reductase • phosphorylation • bisindolylmaleimide I • protein kinase C • regulation • desmosterol • gas chromatography-mass spectrometry

Cholesterol is a vital raw material for the cell, which is toxic in excess and leads to a number of diseases. Thus, cellular cholesterol levels are tightly regulated through a balance of synthesis, uptake, and efflux. The final step in the cholesterol synthetic pathway is catalyzed by the enzyme 3β-hydroxysterol Δ24-reductase (DHCR24). DHCR24 is a 60.1 kDa endoplasmic reticulum membrane-bound oxidoreductase that converts desmosterol to cholesterol by saturating the C-24,25 double bond in the side-chain (1). DHCR24 activity is strictly dependent on nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agent (1), although no consensus sequence for NADPH binding is predicted. However, DHCR24 does contain a highly conserved flavin adenine dinucleotide (FAD) binding domain [amino acids 111-203] (2), characteristic of the well-defined family of FAD-dependent oxidoreductases (3); with reduction of desmosterol dependent on FAD, suggesting functionality of the conserved domain (1). Additionally, a substrate binding domain was predicted in DHCR24 (amino acids 236-491) by homology modeling on an FAD-dependent oxidoreductase, plant cytokinin dehydrogenase (4).

DHCR24 is involved in multiple cellular functions. Related to its role in cholesterol biosynthesis, DHCR24 modulates lipid raft formation, thus facilitating signal transduction and trafficking (5–7). It is involved in regulating oxidative stress (8, 9), and is neuroprotective (5, 10, 11), anti-apoptotic (8, 10), and anti-inflammatory (12, 13). DHCR24 is also implicated in many diseases, such as cardiovascular diseases (12, 14), cancers (e.g., prostate cancer) (15), and hepatitis C (16). Moreover, mutations in DHCR24 underlie the rare autosomal recessive disease, desmosterolosis, whereby patients have elevated desmosterol and lowered cholesterol, resulting in multiple congenital anomalies (17). Specifically, seven missense mutations have been described in desmosterolosis: R94H, R103C, E191K, N294T, K306N, Y471S, E480K (1, 18–21).

Like many cholesterol synthetic genes, DHCR24 is transcriptionally regulated by sterols via the sterol-regulatory element-binding protein-2 transcription factor (22), and we recently identified two sterol-regulatory elements and

Abbreviations: Arg-TLC, argentina-thin-layer chromatography; BIM, bisindolylmaleimide I; CD, methyl-β-cyclodextrin; CHO, Chinese hamster ovary; DHCR24, 3β-hydroxysterol Δ24-reductase; EV, empty vector; FAD, flavin adenine dinucleotide; LPDS, lipoprotein-deficient serum; NBS, newborn bovine serum; PBGD, porphobilogen deaminase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; SIM, single ion monitoring.

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nuclear factor Y sites in the human DHCR24 promoter that mediate this regulation (23). Moreover, DHCR24 is regulated at the transcriptional level by sex steroids (24, 25), adrenocorticotropin hormone (26), thyroid hormone (27), and xenobiotics (28). Epigenetic factors such as methylation and acetylation also regulate DHCR24 expression (29). In contrast, relatively little is known about the posttranscriptional regulation of DHCR24 activity. We recently found that the oxysterol regulator, 24(S),25-epoxycholesterol, inhibits DHCR24 activity with potent effects on cellular cholesterol levels (30). Plant sterols (31) and progesterone and similar progestins (32, 33) similarly inhibit DHCR24 activity. However, the role of posttranslational modifications in DHCR24 regulation, such as phosphorylation, has yet to be investigated. Large-scale proteomic studies have identified phosphorylation sites on DHCR24; T110 (34) and Y507 (35), and there is evidence for additional phosphorylation sites identified by large-scale unpublished proteomics studies [PhosphoSitePlus (36)]. The function of these phosphorylation sites and the kinases/phosphatases involved are currently unknown due to a lack of mechanistic studies.

Cell signaling and phosphorylation is a major mode of regulating cellular processes, and is implicated in various facets of cholesterol homeostasis [reviewed in (37)]. Because DHCR24 catalyzes the ultimate step in cholesterol synthesis, we hypothesized that it is a likely target for feedback regulation via signaling, notably phosphorylation, as this would provide a rapid means of switching cholesterol synthesis on or off. In this study, we examined the effect of known phosphorylation sites on DHCR24 expression and activity. Our data demonstrate that particular putative phosphorylated residues (T110, Y299, and Y507) have significant effects on DHCR24 activity. In addition, inhibiting a major serine/threonine kinase, protein kinase C (PKC), ablated DHCR24 activity, although this effect was independent of T110, the only published serine/threonine phosphorylation site. Together, these results indicate a novel regulatory mechanism for DHCR24 by kinases and phosphorylation.

MATERIALS AND METHODS

Materials

Chinese hamster ovary (CHO)-7 cells (38) were a generous gift from Drs. Michael S. Brown and Joseph L. Goldstein (University of Texas Southwestern Medical Center, Dallas, TX). The Flp-In-CHO cell line (for stable-cell generation) was generated from CHO-7 cells by Gorjana Mitic. HeLaT cells were generously donated by Dr. Noel Whitaker (The University of New South Wales, Sydney, NSW, Australia). SensiMix SYBR No-Rox was from Bio-line (London, UK). Fetal calf serum (FCS) was from Bovogen (East Keilor, VIC, Australia). Amersham Hyperfilm ECL was from GE Healthcare (Bucksinghamshire, UK). Horseradish peroxidase-conjugated AffiniPure donkey anti-mouse IgG antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-V5 antibody, Dulbecco’s Modified Eagle’s Medium-Ham’s Nutrient Mixture F12 (1:1) (DMEM/F12), Lipofectamine RNAiMAX transfection reagent, newborn bovine serum (NBS), Roswell Park Memorial Institute 1640 medium (RPMI), and SuperScript III reverse transcriptase were from Life Technologies (Carlsbad, CA). Akt inhibitor VIII was from Merck (Darmstadt, Germany). Immobilon Western chemiluminescent horseradish peroxidase substrate enhanced chemiluminescent detection system was from Millipore (Billerica, MA). Phusion high-fidelity buffer, Phusion Hot Start II high-fidelity polymerase, and purified BSA were from New England Biolabs (Ipswich, MA). [3H]acetyl sodium salt (specific radioactivity: 55.3 mCi/mmol, 2.046 GBq/mmol) was from Perkin Elmer (Waltham, MA). α-Tubulin antibody, Bis-Tris, bisindolylmaleimide I (BIM), cycloheximide, compatin (mevastatin), desmosterol, H89, primers, mevalonate, MG132 (Z-Leu-Leu-Leu-al), MOPS, N-O-bis-(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA), protease inhibitor cocktail, Ro-318220, siRNA, sodium bisulphite, zinc (II) nitrate, and Tri reagent were from Sigma-Aldrich (St. Louis, MO). [3H]desmosterol was from Avanti Polar Lipids (Alabaster, AL). Phos-tag acrylamide pendant [10 mg supplied in 100 μl 3% (v/v) methanol] was from Wako Pure Chemical Industries (Osaka, Japan). Lipoprotein-deficient serum (LPDS) was prepared from heat-inactivated NBS as previously described (39).

Cell culture and treatments

CHO-7 cells were maintained in 5% (v/v) LPDS/DEME/F12. CHO-7 stable cell-lines were generated and maintained in 5% (v/v) LPDS/DEME/F12 supplemented with 150 μg/ml hygromycin B. HeLaT cells were maintained in 10% (v/v) FCS/RPMI. Where there were treatments, cells were treated in fresh media with various test agents [added in dimethyl sulfoxide or ethanol; desmosterol was complexed to methyl-β-cyclodextrin (CD) according to (40)], as indicated in the figure legends. Within an experiment, the final concentrations of solvent were kept constant between conditions and did not exceed 0.2% (v/v).

Generation of CHO overexpressing cell-lines

Primers were designed to perform megaprimer site-directed mutagenesis (41) on a plasmid encoding the human DHCR24 sequence (accession number NM_014762.3) containing a FRT recombination site (pcDNA5-FRT-DHCR24-V5) (30) to create plasmids encoding DHCR24 phosphorylation mutants T110A, Y299F, Y300F, Y321F, Y507F, and a T110 phosphoryomimetic (T110E). These plasmids were then used to generate stable cell-lines using the CHO-7 Flp-In system, as described in (30). DHCR24 protein expression was screened by Western blotting with the anti-V5 antibody, and stable cells expressing similar levels of DHCR24 were selected and characterized.

The empty vector stable cell-line (CHO-EV) was prepared as described (42), and the human wild-type DHCR24 overexpressing cell-line (CHO-DHCR24-WT) was prepared as described (30).

siRNA transfection

Cells were transfected with 25 nM control or hamster-specific DHCR24 siRNA [target sequence: GAGAGCCACGTGTGAAGCA; designed by Sigma-Aldrich] for 24 h using Lipofectamine RNAiMAX transfection reagent according to the manufacturer’s instructions. The cells were washed with phosphate-buffered saline (PBS), and then refed fresh media with [3H]acetate labeling for argmentation-thin-layer chromatography (Arg-TLC).

Quantitative real-time RT-PCR

Cells were seeded in tripleplate wells per condition as described. Total RNA (1 μg) was reverse transcribed into cDNA using the SuperScript III First Strand cDNA synthesis kit. mRNA levels were determined by quantitative real-time reverse transcription PCR (qRT-PCR) using the Corbett Rotorgene 3000 (Corbett Life
Sciences, Sydney, NSW, Australia) and analyzing using Rotor
Gene version 6.0 (Build 27) (Qiagen, Doncaster, VIC, Australia).
Primers were used to amplify the cDNA of hamster or human
DHCR24 (43) and the housekeeping control, porphobilogen
deaminase (PBGD) (44, 45). Changes in DHCR24 gene expres-
sion levels were normalized to PBDG for each sample by the ΔΔCt
method, and made relative to the CHO-7 cell-line, which was set
to one.

Western blotting

After treatment, cells were harvested in 10% (w/v) SDS sup-
plemented with 5% (v/v) protease inhibitor cocktail. Equal
amounts of protein were mixed with loading buffer (final con-
centration: 50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v)
glycerol, 0.04% (w/v) bromophenol blue, and 1% (v/v) β-
mercaptoethanol), boiled for 5 min, and subjected to SDS-PAGE.
After electrophoresis, the proteins were transferred to a nitrocel-
lulose membrane, blocked for 1 h, incubated with primary an-
ti-V5 antibody (1:5,000) or anti-α-tubulin (1:200,000), and then
further incubated with secondary antibody (1:20,000). The anti-
bodies were visualized by the enhanced chemiluminescent detec-
tion system, and membranes were exposed to Hyperfilm. Proteins
were identified by their predicted molecular mass (α-tubulin, 50
kDa; DHCR24, 60 kDa). Protein band intensities from Western
blots were quantified using densitometry using ImageJ (version
1.47i).

Cholesterol synthesis assay

Cholesterol and desmosterol synthesis were measured as an
indicator of DHCR24 activity using Arg-TLC as described previ-
ously (30). The relative intensities of bands were quantified using
Sciencedlab ImageGauge 4.0 software (Fujifilm).

Phos-tag SDS-PAGE

Phosphorylated proteins were visualized using the phos-tag
SDS-PAGE method described in (46), with modifications. Cells
were seeded in 60 mm or 100 mm dishes, and treated with vari-
est test agents, as indicated in the figure legends. After treat-
ments, cells were washed twice with ice-cold PBS. The cells
were scraped in PBS, then pelleted and lysed in 100 μl modified RIPA
buffer [50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1% sodium
deoxycholate, and 1% Triton X-100 (47)]. The lysates were
passed through a 21 gauge needle 20 times, and centrifuged at
20,000 g at 4°C for 15 min. Equal amounts of protein were mixed
with 0.25 volume 5× loading buffer and boiled for 5 min before
subjecting to phos-tag SDS-PAGE (7.5% separation gel contain-
ing Zn2+-phos-tag complex and a 4% stacking gel), transferred to
nitrocellulose membranes, and Western blotted as indicated in
the figure legends. Purified BSA (0.5 μg) was prepared in the
same volume of modified RIPA buffer containing loading buffer,
and served as a molecular mass marker (66 kDa).

GC-MS

Cells were treated as indicated in the figure legends with or
without 1 μg/ml [3H]desmosterol/CD for 4 h. Cells were har-
vested and lipid extracts were prepared as described in (30).

Sterolized samples were analyzed using a Thermo Trace gas chromato-
graph coupled with a Thermo DSQII mass spectrometer and
Thermo Triplus autosampler (Thermo Fisher Scientific, Waltham,
MA). Samples (1 μl) were injected via a heated (290°C) splitless
inlet into an Rxi-5Sil mass spectrometer with Integra-Guard,
30 m × 0.25 mm, df 0.25 μm film thickness, capillary GC column
(Restek, Waltham, MA). The column oven was initially held at
80°C for 1 min, then heated to 260°C at 8°C min−1, then to
280°C at 10°C min−1, and then to 295°C at 2°C min−1. Finally, the
oven was increased to 305°C at 10°C min−1 and held for 1 min.
Helium was used as a carrier gas at a constant flow (1.3 ml min−1,
with vacuum compensation on).

MS conditions were electron energy 70 eV, ion source tem-
perature 200°C, and transfer line temperature 305°C. The emis-
tion current was set to 130 μA and the detector gain to 3.0 × 106.
Samples were analyzed either in scan mode (35–520 Da, 2.5
scans s−1) to obtain mass spectra for peak identification, or in
single ion monitoring (SIM) mode to measure DHCR24 activity.
Method details and the m/z values of SIM ions monitored for 5α-
cholestanate, [3H]cholesterol, cholesterol, [3H]desmosterol, and
desmosterol were listed in Supplementary Table I. Thermo Xcali-
bur software (version 2.1.0.1140) was used to acquire and process
the data. 5α-Cholestanate (0.1 μg added during cell harvesting)
was used as an internal standard. Peak identification was based
on comparison of retention times with those of standards and
comparison of mass spectra with the Wiley 9/NIST 2011 com-
bined mass spectral library.

Statistical analysis

Statistical differences were determined by the Student’s paired
$ t $ test (two-tailed), where $ P $ values of <0.01 (**) were considered
statistically significant. Statistical analysis was performed in
Fig. 5D using a two-way ANOVA with repeated measures (stability
over time of WT vs. Y299F or Y300F).

RESULTS

PKC inhibition decreases DHCR24 activity

To examine the effect of common protein kinases on
DHCR24 activity, we used inhibitors of protein kinase A
(PKA) [H89 (48)], protein kinase B (PKB) [also called Akt; AktιVIII (49),
and protein kinase C (PKC) [BIM (50)
and Ro-318220 (51)]. CHO-7 cells stably transfected with
epitope-tagged human DHCR24 (30) were radiolabeled
with [14C]acetate, which feeds into the beginning of the
cholesterol synthetic pathway. Cholesterol and desmos-
terol were resolved by Arg-TLC, and radioactive bands
were visualized by phosphorimaging. The cholesterol to
desmosterol ratio was used as an indicator of DHCR24
activity (30).

As shown previously (30), treatment with unlabeled des-
mosterol/CD decreased the cholesterol to desmosterol
ratio, demonstrating competitive inhibition of DHCR24
enzyme. Inhibition of PKA (H89) or PKB (AktιVIII) did not
have a selective effect on DHCR24 activity (Fig. 1A),
although H89 affected cholesterol synthesis on a global level
(supplementary Fig. I). In contrast, PKC inhibitors, BIM
and Ro-318220, reduced cholesterol levels and accumu-
lated desmosterol within 4 h, indicating decreased
DHCR24 activity. BIM had the most robust effect, and abated
DHCR24 activity in a dose-dependent manner (Fig. 1B).
Conversely, PKC stimulation with phorbol myristate ace-
tate increased cholesterol synthesis by $ \sim 30%$ (±5%, $P < 0.01$, Student’s $t$ test). Because inhibition did not affect
DHCR24 protein levels (Fig. 1A), these results suggest that
PKC may influence DHCR24 activity through modulating
its phosphorylation state.
Western blotting, or radiolabeled with [14C]acetate during the indicated concentrations. A, B: Cells were either harvested for treatment for 4 h with BIM at the concentrations. BIM (5 μM), and Western blotting for DHCR24 (V5) and α-tubulin. Western blots are representative of at least two separate experiments. C: Left panel: CHO-7 cells were either harvested for Western blotting or radiolabeled with [14C]acetate during the treatment for Arg-TLC. For Western blotting, whole cell lysates were subjected to SDS-PAGE and Western blotting for DHCR24 (V5) and α-tubulin. Western blots are representative of at least two separate experiments. For Arg-TLC: lipid extracts were separated by Arg-TLC, and bands corresponding to cholesterol (Chol) and desmosterol (Desm) were visualized by phosphorimaging. Arg-TLC (Fig. 1A), competition from unlabeled desmosterol inhibited the conversion of [3H]desmosterol to [3H]cholesterol, resulting in an increase in [3H]dsmosterol (Fig. 2C). BIM treatment had similar effects, strongly inhibiting DHCR24 activity, as evidenced by the [3H]cholesterol to [3H]desmosterol ratio (Fig. 2C).

**PKC inhibition results in desmosterol accumulation**

To confirm the identity of the desmosterol band accumulating with BIM treatment using Arg-TLC, samples were reanalyzed using GC-MS (Fig. 2). Two peaks were identified, where the major peak was cholesterol and a second minor peak, which increased with BIM treatment (Fig. 2A), was identified as desmosterol based on the mass spectra (Fig. 2B). To measure DHCR24 activity, samples were treated with BIM and [2H6]desmosterol. As observed with Arg-TLC (Fig. 1A), competition from unlabeled desmosterol inhibited the conversion of [3H]desmosterol to [3H]cholesterol, resulting in an increase in [3H]desmosterol (Fig. 2C). BIM treatment had similar effects, strongly inhibiting DHCR24 activity, as evidenced by the [3H]cholesterol to [3H]desmosterol ratio (Fig. 2C).

**DHCR24-T110 phosphorylation mutant decreases DHCR24 activity**

From the panel of kinase inhibitors tested, only PKC inhibitors affected DHCR24 activity. Because PKC phosphorylates serine and threonine residues, it is possible that BIM inhibits DHCR24 activity through the only known serine/threonine phosphorylation site, T110 (34). Thus, we mutated this to alanine (T110A; mutant) or glutamic acid (T110E; phosphomimetic), and stably overexpressed these constructs in CHO-7 cells, as we did previously with the generation of the CHO-DHCR24-WT cells (30). DHCR24 mRNA and protein levels between the CHO-DHCR24 cell-lines were comparable (supplementary Fig. II).

To measure the specific effects of the mutants on human DHCR24, endogenous hamster DHCR24 expression was reduced using RNA interference. Transfection of hamster-specific DHCR24 siRNA ablated endogenous hamster DHCR24 mRNA without affecting human DHCR24 expression (Fig. 3A). Thus, transfecting CHO-DHCR24 cells with hamster siRNA reduced background activity in our stable cells, with measured DHCR24 activity attributed to the stably expressed human DHCR24 only.

Using this approach, we examined whether DHCR24-T110 mutants affect DHCR24 activity. As indicated in Fig. 3B, transfecting DHCR24 siRNA into empty vector (EV) cells ablated endogenous DHCR24 activity. Mutating T110 so that it cannot be phosphorylated (T110A), significantly decreased DHCR24 activity by ~60% (Fig. 3B). Furthermore, DHCR24 activity of the phosphomimetic (T110E) mutant was increased relative to T110A, with activity not statistically different from WT cells. These results suggest that phosphorylation at T110 modulates DHCR24 activity.

To confirm that DHCR24 was phosphorylated in our cell system, we employed the phos-tag SDS-PAGE method (46). This system utilizes gels supplemented with phos-tag acrylamide-bound metal complexes, which have affinity for phosphate groups on proteins. This, therefore, slows the migration of phosphorylated species during electrophoresis, producing a mobility shift. As a positive control, we treated cells with a phosphatase inhibitor (Na3VO4) and determined ERK1/2 phosphorylation. Na3VO4 produced two distinct bands when probed with total ERK1/2 antibody, with the bottom band being the nonphosphorylated ERK1/2, as confirmed with phosphorylated ERK1/2 (pERK1/2) antibody (Fig. 1C, left panel). In CHO-DHCR24-WT cells, nontreated cells resulted in multiple slower migrating DHCR24 bands (Fig. 1C, right panel). Interestingly, BIM treatment produced a faster migrating band, suggesting that DHCR24 is constitutively phosphorylated at more than one site.

**Fig. 1.** Effect of kinase inhibitors on DHCR24 activity. A: CHO-7 cells stably overexpressing DHCR24-V5 were seeded and treated for 4 h with desmosterol/CD (Desm/CD) (2 μg/ml), H89 (10 μM), Akt inhibitor VIII (AktVIII) (5 μM), BIM (5 μM), or Ro-318220 (10 μM). B: CHO-7 cells stably overexpressing DHCR24-V5 were seeded, pretreated in NBS, and then treated for 4 h with BIM at the indicated concentrations. A, B: Cells were either harvested for Western blotting, or radiolabeled with [14C]acetate during the treatment for Arg-TLC. For Western blotting, whole cell lysates were subjected to SDS-PAGE and Western blotting for DHCR24 (V5) and α-tubulin. Western blots are representative of at least two separate experiments. For Arg-TLC: lipid extracts were separated by Arg-TLC, and bands corresponding to cholesterol (Chol) and desmosterol (Desm) were visualized by phosphorimaging. Arg-TLC is representative of at least two separate experiments. C: Left panel: CHO-7 cells were seeded and then treated with (+) or without (−) Na3VO4 for 4 h. Right panel: CHO-7 cells stably overexpressing DHCR24-WT were seeded and treated with or without BIM (5 μM) for 4 h. Whole cell lysates were subjected to 7.5% SDS-PAGE supplemented with Zn2+ phos-tag (50 μM), and Western blotted for pERK1/2 and total ERK1/2 (left panel) or DHCR24 (V5; right panel). * denotes ~66 kDa where BSA migrates, and close to the calculated molecular mass of DHCR24, 60 kDa. These results were observed at least twice.

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PKC inhibition decreases DHCR24 activity independently of T110

Next, we determined whether BIM inhibition of DHCR24 activity occurs at T110. Cells were treated in low (LPDS) or high (NBS) cholesterol conditions to exclude any potential effects of cholesterol status. BIM had similar effects in DHCR24-T110A as WT cells: a decrease in DHCR24 activity, with no effect on DHCR24 protein levels (Fig. 4). This indicates that BIM targets a different residue in DHCR24, or that the effect is indirect.

Other DHCR24 phosphorylation sites

Another published phosphorylation site is Y321 (35) and PhosphoSitePlus (36) give evidence for three other phosphorylated tyrosine residues, Y299, Y300, and Y507. Thus, we similarly generated DHCR24 stable cell-lines containing these mutants, and characterized mRNA and protein expression. DHCR24 protein correlated with mRNA expression (supplementary Fig. III), with the exception of the DHCR24-Y299F and DHCR24-Y300F mutants. Compared with the DHCR24-WT, the DHCR24-Y299F stable cells contained lower mRNA expression while having comparable protein levels. Conversely, the DHCR24-Y300F stable cells had similar levels of mRNA to the DHCR24-WT stable cells, while containing less protein.

Examining the protein stability of DHCR24-Y299F and DHCR24-Y300F

Y299 and Y300 are neighboring residues, yet phosphorylation mutants at these sites have vastly different effects on DHCR24 mRNA and protein expression, suggesting that mutating DHCR24 at these residues may alter protein stability. In addition, there is evidence for DHCR24 ubiquitination (36), including at K301 (52), which is adjacent to Y299 and Y300. Therefore, we compared DHCR24 protein...
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Effect of tyrosine mutants on DHCR24 activity

Next, we determined whether DHCR24 stable cell-lines containing mutant tyrosine residues affected DHCR24 activity. Mutating Y299 and Y507 significantly decreased DHCR24 activity by ~40% and ~60%, respectively, while mutating Y300 and the published site, Y321, had no effect (Fig. 6). Thus, Y299 and Y507, but not Y300 and Y321, may be phosphorylation sites that regulate DHCR24 activity.

DISCUSSION

We discovered a novel mode of regulation of a key protein in cholesterol synthesis, DHCR24. Recent studies on DHCR24 have identified a number of regulatory mechanisms, primarily at the transcriptional level and particularly via feedback regulation by sterol/steroid molecules, with only a few at the posttranslational level. Regulation by posttranslational modifications, such as phosphorylation, has yet to be fully investigated, despite evidence that DHCR24 is phosphorylated at multiple sites (36), which is in line with our phos-tag SDS-PAGE results (Fig. 1C).
BIM and Ro-318220 are therefore relatively specific inhibitors of the PKC family, with BIM reducing DHCR24 activity in a dose-dependent manner from concentrations as low as 0.1 μM. Thus, our results are consistent with the involvement of PKC in modulating DHCR24 activity. However, further experiments should confirm this, and determine which of the 10 distinct PKC isoforms is involved.

We then determined whether BIM inhibition of DHCR24 activity could be negated by mutating the only published serine/threonine phosphoresidue in DHCR24, T110. T110 is also predicted to be a low stringency PKC substrate by a matrix-based prediction program, Scansite (55) [see supplementary Table II for a list of sites examined here]. However, the BIM inhibitory effect was still observed in the DHCR24 T110 mutant cell-line (CHO-DHCR24-T110A), indicating that PKC does not phosphorylate DHCR24 at T110. This suggests that BIM may be affecting DHCR24 directly through an as yet unidentified phosphorylation site(s), or indirectly. Future work should involve testing other putative sites, as predicted by Scansite, for PKC phosphorylation and effect on DHCR24 activity.

Another aim of this work was to characterize the known phosphorylation sites in DHCR24 and examine their effects on DHCR24 expression and activity. We developed a novel method for measuring DHCR24 activity in cell culture, by stably expressing human DHCR24 in hamster

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**Fig. 6.** Effect of mutating tyrosine residues on DHCR24 activity. CHO-7 cells stably overexpressing EV or DHCR24 (WT, Y299F, Y300F, Y321F, and Y507F) were seeded and transfected with control or hamster-specific DHCR24 siRNA (25 nM) for 24 h. The cells were washed with PBS and refed fresh media overnight, and then radiolabeled with [14C]acetate in fresh media. Lipid extracts were separated by Arg-TLC, and bands corresponding to cholesterol (Chol) and desmosterol (Desm) were visualized by phosphorimaging. Arg-TLC is representative of four separate experiments, and the relative cholesterol to desmosterol ratio was quantified by densitometry, normalized to protein expression (supplementary Fig. IIIB), and then normalized to the DHCR24 siRNA transfected CHO-DHCR24-WT condition, which has been set to one. **P < 0.01 compared with the DHCR24 siRNA transfected CHO-DHCR24-WT condition. Data are mean ± SEM (n = 4).**

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One aim of this work was to identify protein kinase(s) involved in DHCR24 phosphorylation by looking at common protein kinases, using a panel of kinase inhibitors. While inhibiting serine/threonine kinases did not affect DHCR24 protein expression, inhibition of PKC (using BIM and Ro-318220) markedly decreased desmosterol to cholesterol conversion. A potential caveat when studying PKC with pharmacological inhibitors is the issue of specificity (53). For example, BIM and Ro-318220 have been shown to exhibit comparable inhibition of 90 kDa and 70 kDa ribosomal S6 kinase and PKC in vitro, but in cell culture, at least 10 times higher concentrations are required to inhibit the S6 kinases (54). BIM and Ro-318220 are therefore relatively specific inhibitors of the PKC family, with BIM reducing DHCR24 activity in a dose-dependent manner from concentrations as low as 0.1 μM. Thus, our results are consistent with the involvement of PKC in modulating DHCR24 activity. However, further experiments should confirm this, and determine which of the 10 distinct PKC isoforms is involved.
Signaling regulates DHCR24 activity

Interactions with the negatively-charged phosphate groups in FAD. Further work testing FAD binding affinity in DHCR24-T110A is required to consolidate this hypothesis.

The other published phosphorylation site was a tyrosine residue (Y321), with three other tyrosine residues implicated. We created phosphorylation mutants by substituting the tyrosine with phenylalanine (Y to F), and stably expressed these in CHO cells. Similar to T110A, the Y299F and Y507F mutants significantly decreased DHCR24 activity, with a ~60% reduction in activity observed for Y507F relative to DHCR24-WT (Fig. 6). Y507 is a highly conserved residue at the extreme C-terminal end of DHCR24 (Fig. 7), which lies within a small region predicted to interact with the cofactor FAD (4).

The kinases/phosphatases responsible for T110, Y299, and Y507 phosphorylation remain elusive and require further investigation. In this study, we have excluded the effects of PKA, PKB, and PKC (Figs. 1A, 4), as well as ERK1/2, PI3K, mTOR, JAK2, SHP1/2, and PTP1B (data not shown). We have also attempted to confirm these phosphorylation sites in our cell system by liquid chromatography-tandem mass spectrometry. While we had reasonable sequence coverage for DHCR24 (up to 32%), we were unable to detect the required peptides, as determining phosphorylation with this approach remains challenging in our hands (57). Nevertheless, the phosphorylation sites examined in this study were originally identified in large-scale phosphoproteomics studies, with T110 being a published phosphorylation site (34), as well as being identified in Cell Signaling Technology MS/MS studies. As discussed, T110 is very likely physiologically relevant, as the phosphorylation mutant decreased and the phosphomimetic restored DHCR24 activity. While Y507 is not a published phosphorylation site,
PhosphoSitePlus indicated this site to be a phosphorylated residue in 12 data sets (36), increasing confidence that it is a bona fide phosphorylation site. Furthermore, our phos-tag result suggested that DHCR24 is phosphorylated at more than one site in our cell system (Fig. 1C).

Although this study focused on potential posttranslational modification by phosphorylation, there is a vast amount of evidence that DHCR24 is ubiquitinated. At present, there are 11 identified ubiquitination sites (9 published (52, 58–60) and 2 from PhosphoSitePlus (36)), suggesting that DHCR24 may be regulated by proteasomal degradation. One such site, K301 (52), is a highly conserved residue that neighbors putative phosphosidues Y299 and Y300 (Fig. 7). Although the mutants are in close proximity, they have differing effects on DHCR24 mRNA and protein expression, with DHCR24Y300F levels exhibiting a striking decrease from mRNA to protein (supplementary Fig. III). Therefore, we suspected that this mutant might be subject to proteasomal degradation. However, both DHCR24Y299F and DHCR24Y300F caused only minor destabilization that cannot explain the observed mRNA and protein levels, which are not due to differences in cell-lines, as we used a Flp-In system which introduces only one copy of a gene into each cell, and different clones gave the same effect (data not shown). Notably, DHCR24WT was very stable under the conditions tested (Fig. 5).

There is emerging evidence that other enzymes in the cholesterol synthetic pathway besides HMG-CoA reductase (the classic rate-limiting step) are potential control points for rapid regulation of cholesterol synthesis (61), such as squaleno monooxygenase (62). DHCR24 is a prime candidate (61) due to its position in the Bloch pathway as the final enzyme. Thus, regulating DHCR24 by phosphorylation would allow for a rapid means of attenuating cholesterol synthesis, with previous studies already demonstrating its ability for rapid inhibition by multiple feedback mechanisms (30, 32). Furthermore, modulating DHCR24 activity alters levels of desmosterol, a strong liver X receptor ligand (14, 63), further reducing cellular cholesterol status. Collectively, this study has demonstrated that signaling and phosphorylation have functional effects on DHCR24. Further work is required to investigate the precise kinase(s) responsible for the phosphorylation sites investigated in this study, as well as the phosphorylation site(s) required for PKC’s action.44

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