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23. CV-1 cells were cotransfected with a rat FXR expression plasmid, a luciferase reporter construct containing five copies of an IR-1 response element, and a β-galactosidase (β-Gal) expression vector as a marker as described (*4*, *13*). Transfected cells were treated with various compounds (Sigma) for 36 hours and then harvested for luciferase assay. For HepG2 cells cotransfected with human FXR, the luciferase reporter plasmid contained three copies of the IR-1 (AGGTCAATGACCT), and cells were treated for 20 hours with compounds before being harvested. Cotransfections with Gal4-receptor chimeras included a luciferase reporter gene (G5-Luc) containing five copies of the Gal4 DNA binding site. Transfection data were normalized to β-Gal, are expressed relative to ethanol solvent controls as fold induction or relative light units (RLUs), and represent triplicate assays ± SD.
24. HEK-293 cells were transfected with plasmids expressing the chimeric proteins Gal4–SRC-1 (amino acids 583 through 783), FXR (amino acids 105 through 472)–VP16, and the G5-Luc reporter. Luciferase activity was measured as in (*23*).
25. The FXR LBD (amino acids 105 through 472) was fused to the COOH-terminus of glutathione S-transferase (GST), and the resultant GST-FXR protein was expressed in *Escherichia coli* and then purified on glutathione beads. For the FRET assay, a europium-labeled antibody to GST [anti-GST–(Eu)] (Wallac, Gaithersburg, MD) was used to tag GST-FXR. SRC-1 (amino acids 595 through 822) was tagged with hexahistidine, expressed in *E. coli*, purified by metal ion chromatography, biotinylated, and labeled with fluorophore allophycocyanin (APC) (Wallac) conjugated to streptavidin. FRET occurs in solution when ligand-mediated changes in the conformation of FXR increase its affinity for SRC-1, resulting in energy transfer from europium (337 nm excitation and 620 nm emission) to APC (620 nm excitation and 665 nm emission). Results are expressed as a ratio of APC to europium fluorescence (665 nm/620 nm). To each well of a black polypropylene 96-well plate was added 10 nM GST-FXR, 100 nM biotin–SRC-1, anti-GST–(Eu) (0.2 μg/ml), APC-streptavidin (1 μg/ml), and the indicated compound in 100 μl of buffer [100 mM Hepes (pH 7.6), 0.125% CHAPS, and 125 mM NaF]. The reaction was mixed and incubated for 12 hours at 4°C, and fluorescence was measured on a Victor II plate reader (Wallac). For ELISA, 1.5 μM biotin-labeled peptide (amino acid sequence Ile-Leu-Arg-Lys-Leu-Leu-Gln-Glu) was incubated with 100 nM GST-FXR and the indicated compound in 100 μl of buffer [25 mM tris-HCl (pH 7.4) and 150 mM NaCl] in a 96-well plate for 1 hour. The plate was washed and incubated with rabbit antibody to GST, and GST-FXR protein bound to streptavidin was quantitated with a horseradish peroxidase-labeled antibody to rabbit.
26. CV-1 cells were cotransfected as in Fig. 1 with rat FXR and RXRα expression plasmids and with the indicated luciferase reporter genes. To create the reporter genes, the first 1031 bp (pIBABP$_{1031}$-Luc) or 496 bp (pIBABP$_{496}$-Luc) of the mouse I-BABP gene promoter (*21*) were amplified by polymerase chain reaction (PCR) from mouse genomic DNA and ligated into a Luc reporter plasmid (*13*). The mutant reporter (pIBABP$_{mut\text{-}142}$-Luc) was made from the pIBABP$_{496}$-Luc reporter by site-directed mutagenesis within the I-BABP promoter sequence −142 to −130 (Fig. 2A), which converts nucleotides AGGTGAATAACCT to ACCTGAATAAGGT.
27. Human Cyp7a mRNA was quantitated from HepG2 cells that were treated with the indicated compounds using a TaqMan One Step Gold reverse transcriptase (RT) PCR kit (Applied Biosystems/Perkin Elmer). The Cyp7a primers used were CYP7-78: 5′-TGATTTGGGGGATTGCTATA; CYP7-178: 5′-CATACCTGGGCTGTGCTCT; and CYP7-132(FAM): 5′- (6-FAM) TGGTTCACCCGTTTGCCTTCTCCT (TAMRA). Analysis was performed in triplicate parallel assays.

28. We gratefully acknowledge the late Kazuhiko Umesono, whose pioneering work in the nuclear receptor field inspired much of this work. We thank A. Bronson, J. Bembenek, T. Lu, J. Wu, R. Daly, and L. Miao for reagents, technical support, and helpful discussions; D. Russell for the human Cyp7a promoter and critical comments; and C. Weinberger for rat FXR. M.M. and J.J.R. are associates and D.J.M is an investigator of the Howard Hughes Medical Institute. D.J.M. is supported by a grant from the Robert A. Welch Foundation.

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Bile Acids: Natural Ligands for an Orphan Nuclear Receptor

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Bile acids regulate the transcription of genes that control cholesterol homeostasis through molecular mechanisms that are poorly understood. Physiological concentrations of free and conjugated chenodeoxycholic acid, lithocholic acid, and deoxycholic acid activated the farnesoid X receptor (FXR; NR1H4), an orphan nuclear receptor. As ligands, these bile acids and their conjugates modulated interaction of FXR with a peptide derived from steroid receptor coactivator 1. These results provide evidence for a nuclear bile acid signaling pathway that may regulate cholesterol homeostasis.

Cholesterol homeostasis is achieved through the coordinate regulation of dietary cholesterol uptake, endogenous biosynthesis, and the disposal of cholesterol in the form of bile acids. Bile acids are not simply metabolic by-products, but are essential for appropriate absorption of dietary lipids and also regulate gene transcription. Among the genes regulated by bile acids are cholesterol 7α-hydroxylase (Cyp7a), the rate-limiting enzyme in bile acid biosynthesis (*1*), and the intestinal bile acid–binding protein (I-BABP), a cytosolic protein that serves as a component of the bile acid transport system in the ileal enterocyte (*2*). I-BABP gene expression is induced preferentially by chenodeoxycholic acid (CDCA) relative to other more hydrophilic bile acids (*3*).

To examine whether CDCA mediates its transcriptional effects through an orphan member of the steroid-retinoid-thyroid hormone receptor family (*4*), we used a chimeric receptor system in which the putative ligand-binding domain (LBD) of the human orphan receptor is fused to the DNA binding domain of the yeast transcription factor GAL4 (*5*). In CV-1 cells, CDCA selectively activated FXR [NR1H4] (Fig. 1), an orphan nuclear receptor expressed predominantly in the liver, kidney, intestine, and adrenals (*6, 7*). This strong activation by CDCA was unanticipated because FXR responds to high concentrations of farnesoids (*6*) and retinoids (*8*).

To further investigate the structure-activ-

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ity relation of FXR activation, we tested a number of naturally occurring cholesterol metabolites, including bile acids (Fig. 2A), oxysterols and steroids, for their ability to activate full-length human or full-length murine FXR in CV-1 cells. CDCA activated both the human and mouse FXR (Fig. 2B). Dose response analysis showed that although some activation was seen at 3.3 μM, greater activation was observed at 100 μM CDCA (Fig. 2C). FXR was also activated by the secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA), although these compounds were less efficacious than CDCA (Fig. 2, B to E). A similar activation pattern was observed in various cell lines, including *Drosophila*-derived S2 cells, indicating that CDCA, LCA, and DCA do not require any specialized metabolic conversion to activate FXR (*9*). Urso-DCA, the 7β-hydroxy epimer of CDCA, and cholic acid (CA), which differs from CDCA by only the addition of a hydroxyl group at the 12α position, were inactive on FXR (Fig. 2B). In addition, no activation of FXR was seen with either α- or β-muricholic acid (MCA), the glycine or taurine conjugates of bile acids, oxysterols, farnesol, or other products derived from the mevalonate pathway (Fig. 2, B and F). Thus, both the 5β-cholanoic acid backbone and stereochemistry of the hydroxyl groups in CDCA are critical for optimal FXR activation.

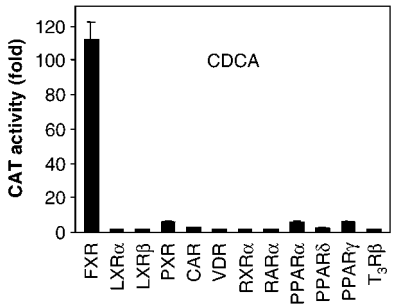

Fig. 1. CDCA selectively activates FXR. CV-1 cells were cotransfected with various nuclear receptor-GAL4 chimeras (*22*) and the reporter plasmid $(UAS)_5$-tk-CAT (*5*). Cells were treated with 100 μM CDCA. Cell extracts were subsequently assayed for chloramphenicol acetyltransferase (CAT) activity (*5*). Data are expressed for each receptor as fold induction of CAT activity relative to vehicle-treated cells and represent the mean of three data points ± SD.

Bile acids are usually found conjugated to glycine or taurine, a derivative of cysteine. Cells require the presence of an active bile acid transporter for uptake of these conjugated derivatives (*10*). To test whether conjugated bile acids would also activate FXR, we coexpressed the human ileal bile acid transporter (IBAT) with FXR in CV-1 cells (*11*). FXR was strongly activated by 3 μM of the taurine or glycine conjugates of CDCA, LCA, and DCA (Fig. 2G). Weaker activation was seen with the conjugated forms of CA, and tauro-MCA was inactive (Fig. 2G). These data indicate that FXR can be activated by conjugated bile acids in tissues that express bile acid transporters such as the terminal ileum, liver, and kidney. The relation between the chemical structure of bile acids and their activation of FXR is in close agreement with the reported effects of bile acids on induction of I-BABP expression in Caco-2 cells and inhibition of Cyp7a expression in hepatocytes (*3, 12*)

Coactivator proteins interact with nuclear receptors in a ligand-dependent manner and augment transcription (*13*). A short amphipathic α-helical domain that includes the amino acid motif LXXLL (L is Leu and X is any other amino acid) serves as the interaction interface between these coactivator molecules and the ligand-dependent activation function (AF-2) located in the COOH-terminus of the nuclear receptor LBD (*14*). This AF-2 function of FXR was essential for response to bile acids (*8, 9*). To test whether CDCA and its conjugates would induce a conformation of FXR that favors coactivator binding, we established a cell-free ligand-sensing assay using fluorescence resonance energy transfer (FRET) to monitor allosteric interaction of the steroid receptor coactivator–1 (SRC-1) with the receptor. The use of FRET to monitor macromolecular complex

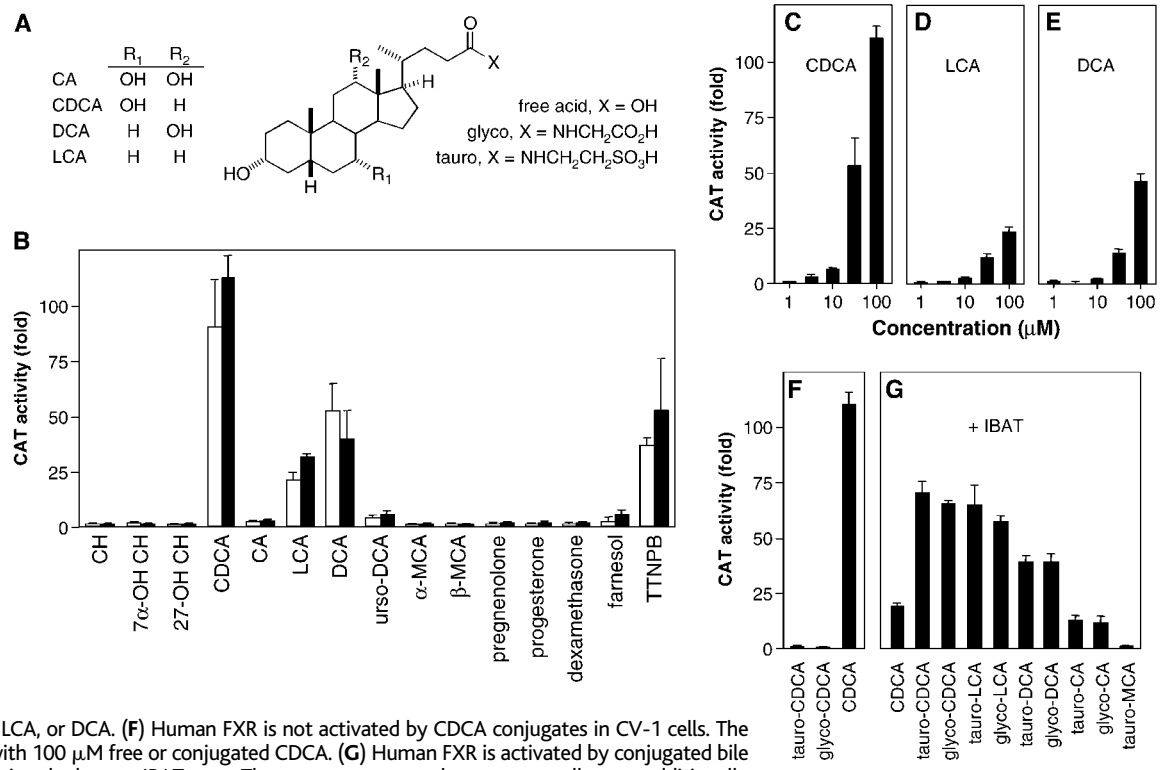

Fig. 2. Activation of FXR by bile acids. (**A**) Chemical structures of major human bile acids. (**B**) Full-length human (filled bars) and full-length murine (open bars) FXR are activated by CDCA, LCA, and DCA. CAT assays were performed with extracts of CV-1 cells transfected with expression plasmids for human or murine FXR, human RXRα, and the FXREhsp27-tk-CAT reporter plasmid (*8, 23*). Cells were treated with 100 μM of the indicated bile acid or farnesol, or 10 μM of the indicated steroid or TTNPB. Cholesterol, CH. (**C** to **E**) Dose response analysis of bile acids on human FXR. The assays were run as above with 1, 3.3, 10, 33, or 100 μM of CDCA, LCA, or DCA. (**F**) Human FXR is not activated by CDCA conjugates in CV-1 cells. The assay was run as above with 100 μM free or conjugated CDCA. (**G**) Human FXR is activated by conjugated bile acids in CV-1 cells expressing the human IBAT gene. The assay was run as above except cells were additionally transfected with an expression plasmid for the human IBAT gene (pCMV-HISBT) and treated with 3 μM of the indicated bile acid. Data are expressed as fold induction of CAT activity relative to vehicle-treated cells and represent the mean of three data points ± SD (*5*).

formation is well established, particularly for immunoassays (*15*), and this detection methodology has recently been extended to characterize ligand binding to nuclear receptors (*16*). The LBD of human FXR was labeled with the fluorophore allophycocyanin and incubated with a peptide derived from the second LXXLL motif of SRC1 (amino acids 676 to 700) that was labeled with europium chelate. CDCA and the corresponding glycine and taurine conjugates increased the interaction between FXR and the SRC1 peptide as determined with time-resolved FRET (Fig. 3A). Dose response analysis showed that CDCA, glyco-CDCA, and tauro-CDCA increased the amount of SRC1 peptide bound to the FXR LBD with half-maximal effective concentration (EC_{50}) values ranging from 4.5 μM for CDCA to 10 μM for the conjugated forms (Fig. 3B and Table 1). These values are well within the physiological range of the concentrations of these bile acids in both the liver and intestine (*17*).

Although LCA, DCA, and (*E*)-[(tetrahydrotetramethylnaphthalenyl)propenyl]benzoic acid (TTNPB) activate FXR in the cell-based reporter assay (Fig. 2B), they did not promote interactions between the FXR LBD and SRC1 in the FRET ligand-sensing assay (Fig. 3A). However, when these compounds were assayed in the presence of 50 μM CDCA, they disrupted the CDCA-FXR-SRC1 complex in a dose-dependent fashion (Fig. 3C and Table 1). Thus, CDCA, LCA, DCA, and TTNPB are ligands for FXR. Similarly, the taurine and glycine conjugates of LCA decreased the fluorescence signal, indicating displacement of CDCA from FXR with IC_{50} values of 3.8 and 4.7 μM, respectively (Table 1). The conjugated forms of DCA and MCA caused a small decrease in fluorescence at the highest concentration tested, and no effect was obtained with the conjugated forms of CA and urso-DCA [Table 1 and (*18*)].

We conclude from several lines of evidence that the orphan nuclear receptor FXR can act as a nuclear receptor for bile acids. First, FXR is most abundantly expressed in liver, intestine, and kidney, tissues that are exposed to significant bile acid fluxes, and that express bile acid transporters. Furthermore, CDCA, LCA, DCA, and their conjugated derivatives bind to FXR at concentrations consistent with those found in tissues and known to regulate gene transcription. Finally, these bile acids are highly efficacious activators of FXR in our cell-based reporter assays. In the accompanying paper, Makishima *et al*. report the effects of FXR on I-BABP and Cyp7a gene transcription (*19*). With the conjugated forms of the bile acids, activation is only observed in cells that express a bile acid transporter. Thus, the conjugated derivatives, which account for ~98% of all bile acids in human bile, are likely to represent natural FXR ligands in tissues that express bile acid transporters, whereas the unconjugated forms may function as ligands in tissues that do not express these transport proteins. The identification of bile acids as natural FXR ligands suggests that these compounds may have important and unexpected functions in mammalian physiology (*20*). The identification of FXR target genes should provide important insights into these functions.

Table 1. Potency of bile acids for binding to FXR as determined in the cell-free ligand-sensing assay (*25*). The indicated values for CDCA and its conjugates are EC_{50}'s derived from dose response analysis as described in Fig. 3B. The indicated values for other bile acids and their conjugates are half-maximal inhibitory concentrations (IC_{50}'s) derived from dose response analysis as described in Fig. 3C.

Bile acid	Free acid	Glycine conjugate	Taurine conjugate
CDCA	4.5 μM	10 μM	10 μM
CA	>1000 μM	>1000 μM	>1000 μM
LCA	3.8 μM	4.7 μM	3.8 μM
DCA	100 μM	≥500 μM	≥500 μM
MCA	>1000 μM	Not tested	≥500 μM

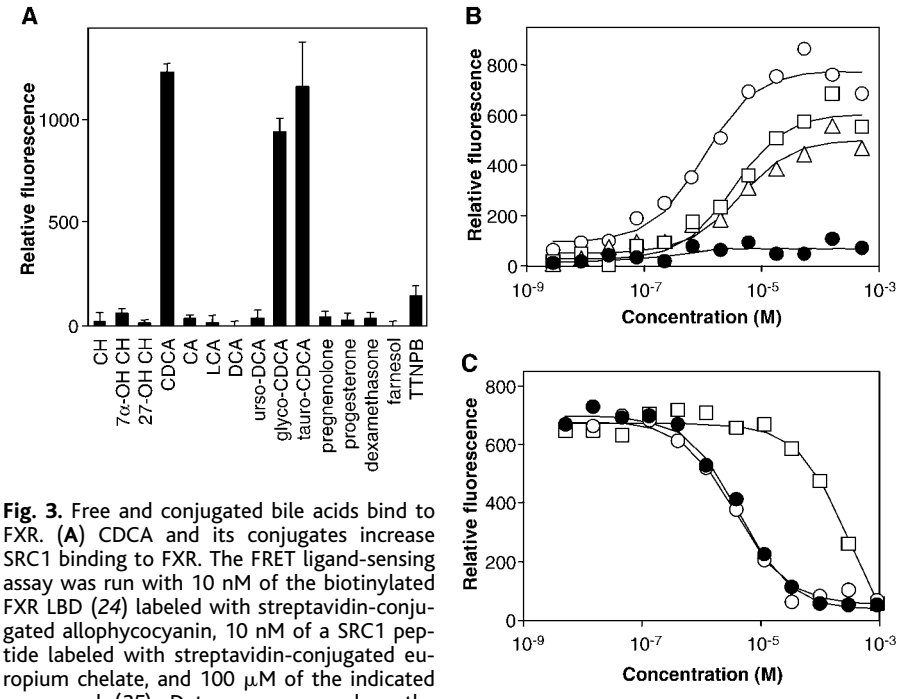

Fig. 3. Free and conjugated bile acids bind to FXR. (**A**) CDCA and its conjugates increase SRC1 binding to FXR. The FRET ligand-sensing assay was run with 10 nM of the biotinylated FXR LBD (*24*) labeled with streptavidin-conjugated allophycocyanin, 10 nM of a SRC1 peptide labeled with streptavidin-conjugated europium chelate, and 100 μM of the indicated compound (*25*). Data are expressed as the means ± SD derived from three independent experiments. (**B**) Dose response analysis of CDCA binding to FXR. The FRET ligand-sensing assay was run in the presence of increasing concentrations of CDCA (open circles), glyco-CDCA (open triangle), tauro-CDCA (open boxes), or cholic acid (closed circles). (**C**) LCA, DCA, and TTNPB compete with CDCA for FXR binding. FRET ligand-sensing assays were run in the presence of 50 μM CDCA and increasing concentrations of LCA (open circles), DCA (open boxes), and TTNPB (closed circles).

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21. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F,

Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

22. Expression plasmids for the human nuclear receptor–GAL4 chimeras were generated by amplification of the cDNA encoding the putative LBDs and insertion into a modified pSG5 expression vector (Stratagene) containing the GAL4 DNA binding domain (amino acids 1 to 147) and the simian virus 40 (SV40) Tag nuclear localization signal (*5*).
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24. DNA encoding the human FXR LBD (amino acids 222 to 472; GenBank U68233) was inserted into the pRSET-A expression vector (Invitrogen) and expressed in bacteria. The transformed cells were grown for 12 hours at 25°C, cooled to 9°C, maintained at this temperature until they reached a cell density, as measured by optical density (OD) at 600 nm, of OD_{600} = 14 , induced with 0.25 mM isopropyl β-D-thiogalactoside to a final cell density of OD_{600} = 16, and harvested by centrifugation. Filtered bacterial lysate was applied to an affinity column of Ni^{2+}-charged chelation Sepharose (Amersham Pharmacia Biotechnology, 25 mM tris-HCl, pH 7.2, 150 mM NaCl, and 50 mM imidazole). Protein was eluted with 365 mM imidazole after washing with buffer containing 95 mM imidazole, and further purified by size exclusion chromatography with Superdex S-75 resin (Amersham Pharmacia Biotechnology). The FXR LBD was biotinylated with NHS-LC-biotin reagent (Pierce).
25. The FRET ligand-sensing assay was performed by incubating 10 nM of the biotinylated FXR LBD that was labeled with streptavidin-conjugated allophycocyanin (Molecular Probes) and 10 nM of the SRC1 peptide [amino acids 676 to 700, 5′-biotin-CPSSHSSLTERHKIL-HRLLQEGSPS-$CONH_2$] (*21*) (SynPEP) that was labeled with streptavidin-conjugated europium chelate (Wallac), in 50 mM tris pH 8, 50 mM KCl, 0.1 mg/ml bovine serum albumin, 1 mM EDTA, and 10 mM dithiothreitol, in the presence of test compound for 2 hours at 22°C. Data were collected with a Wallac Victor fluorescence reader in a time-resolved mode. The relative fluorescence was measured at 665 nM, and the indicated values were calculated by subtracting the fluorescence obtained in the absence of test compound from the value obtained in the presence of test compound. Values are expressed as the means ± SD derived from three independent experiments.
26. We thank P. A. Dawson for providing the IBAT expression plasmid pCMV-HISBT, B. W. O'Malley, S. Y. Tsai, M.-Y. Tsai, and M. C. Lewis for critically reading the manuscript. Supported in part by NIH grant RO1 DK53366 to D.D.M. and National Institute of Diabetes and Digestive and Kidney Diseases grant F32 DK09793 to A.M.Z.

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Modulation of Polyketide Synthase Activity by Accessory Proteins During Lovastatin Biosynthesis

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Polyketides, the ubiquitous products of secondary metabolism in microorganisms, are made by a process resembling fatty acid biosynthesis that allows the suppression of reduction or dehydration reactions at specific biosynthetic steps, giving rise to a wide range of often medically useful products. The lovastatin biosynthesis cluster contains two type I polyketide synthase genes. Synthesis of the main nonaketide-derived skeleton was found to require the previously known iterative lovastatin nonaketide synthase (LNKS), plus at least one additional protein (LovC) that interacts with LNKS and is necessary for the correct processing of the growing polyketide chain and production of dihydromonacolin L. The noniterative lovastatin diketide synthase (LDKS) enzyme specifies formation of 2-methylbutyrate and interacts closely with an additional transesterase (LovD) responsible for assembling lovastatin from this polyketide and monacolin J.

Lovastatin is an inhibitor of the enzyme (3*S*)-hydroxymethylglutaryl–coenzyme A (HMG-CoA) reductase that catalyzes the reduction of HMG-CoA to mevalonate, a key step in cholesterol biosynthesis. This activity confers on lovastatin its medicinally important antihypercholesterolemic activity and other potentially important uses (*1*). It is a secondary metabolite from the filamentous fungus *Aspergillus terreus* [American Type Culture Collection (ATCC) 20542] and has been shown to be derived from acetate via a polyketide pathway (*2*). Polyketide biosynthesis in bacteria and fungi is related to fatty acid metabolism but differs in that some of the reduction or dehydration reactions catalyzed by a polyketide synthase (PKS) can be suppressed at specific biosynthetic steps. Substrates besides acetyl-CoA and malonyl-CoA can be used by PKSs to assemble the carbon chain. These attributes result in a much wider range of possible products than fatty acid metabolism, which together with post-PKS modifications provide a very large family of often biologically active secondary metabolites (*3*).

Microbial polyketides are known to be assembled in three different ways. Bacterial PKSs consist of either the modular (type I) or iterative (type II) systems. In a type I PKS, such as that involved in the biosynthesis of erythromycin A (*4*), one distinct group of active sites, called a module (*5*), on a single polypeptide is used to initiate or extend the carbon chain. The active sites present in each module determine the choice of extender unit plus the level of reduction or dehydration for that particular extension cycle, and the number of modules in the polypeptides constituting the PKS determines the length of the polyketide carbon chain. In contrast, for type II PKS systems each activity is encoded by a separate enzyme that is used several times in the biosynthesis of the typically aromatic product [for example, actinorhodin, doxorubicin, and tetracenomycin C (*6*)].

Fungal PKS systems fall into a third group, iterative type I PKSs. Many fungal PKSs make polyketides similar to those produced by the bacterial type II class: polycyclic aromatic compounds, such as 1,3,6,8-tetrahydroxynaphthalene and 6-methylsalicylic acid, whose biosynthesis is primarily an iterative process involving no reduction and dehydration steps (or only one such step). Mammalian and some microbial fatty acid synthases (FASs) are closely related to this class of PKS, having the same order of domains and an iterative nature (*7*). Fungal PKSs also make nonaromatic reduced compounds such as lovastatin (also called mevinolin or monacolin K), brefeldin A (*8*), and T-toxin (*9*) (Fig. 1A). All of these metabolites are derived from polyketide chains that vary in their state of reduction and dehydration, as well as length. How this is accomplished has been a mystery because it is not obvious how a single set of the activities typically found in an iterative type I PKS can make the choice of oxidation level at each chain-extending condensation.

We report here that the interaction of the two fungal PKSs involved in lovastatin biosynthesis with other enzymes of the pathway seems to modulate their overall activity, apparently endowing one iterative PKS with the ability to discriminate between carbon chain intermediates at different stages of assembly and possibly causing the other PKS to behave noniteratively, like a bacterial modular PKS.

In the lovastatin pathway (Fig. 1B), the

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Bile Acids: Natural Ligands for an Orphan Nuclear Receptor

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Editor's Summary