Differential Gene Regulation of StarD4 and StarD5 Cholesterol Transfer Proteins

ACTIVATION OF StarD4 BY STEROl REGULATION ELEMENT-BINDING PROTEIN-2 AND StarD5 BY ENDOPLASMIC RETICULUM STRESS

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The StarD4 and StarD5 proteins share ~30% identity, and each is a steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain. We previously showed StarD4 expression is sterol-repressed, consistent with regulation by sterol regulatory element-binding proteins (SREBPs), whereas StarD5 is not sterol-regulated. Here we further address the regulation and function of StarD4 and StarD5. Unlike STAR, the START family prototype, StarD4 and StarD5 were not induced by steroidogenic stimuli in Leydig cells. However, StarD4 and StarD5 showed StAR-like activity in a cell culture steroidogenesis assay, indicating cholesterol transfer. In transgenic mice expressing active SREBPs, StarD4 was predominantly activated by SREBP-2 rather than SREBP-1a. The mouse and human StarD4 proximal promoters share ~70% identity, including several potential sterol regulatory elements (SREs). Reporters driven by the StarD4 promoter from either species were transfected into NIH-3T3 cells, and reporter activity was highly repressed by sterols. Site-directed mutagenesis of potential SREs identified a conserved functional SRE in the mouse (TCGGTCCAT) and human (TCATGTTCAT) promoters. StarD5 was not sterol-repressed via SREBP-2 nor was it sterol-activated via liver X receptors (LXRs). Even though StarD4 and StarD5 were not LXR targets, their overexpression stimulated LXRs reporter activity, suggesting roles in cholesterol metabolism. StarD5 expression increased 3-fold in free cholesterol-loaded macrophages, which activate the endoplasmic reticulum (ER) stress response. When NIH-3T3 cells were treated with agents to induce ER stress, StarD5 expression increased 6–8-fold. Because StarD4 is regulated by sterols via SREBP-2, whereas StarD5 is activated by ER stress, they likely serve distinct functions in cholesterol metabolism.

StarD4 and StarD5 belong to the family of StAR-related lipid transfer (START) proteins, which are implicated in lipid transport, metabolism, and signaling (1). A START domain is an ~210-amino acid globular protein domain with an internal hydrophobic cavity for lipid binding. Based on phylogenetic analysis of the START family, StarD4, StarD5, and StarD6 form a subfamily most closely related to the cholesterol binding START domains of the steroidalogenic acute regulatory protein (StAR/StarD1) and MLN64 (StarD3) (2). StarDAR delivers cholesterol to mitochondria in steroidogenic cells (3), whereas MLN64 has been implicated in cholesterol movement from endosomes (4). Other START proteins like phosphatidylcholine transfer protein (PCTP/StarD2) (5) and ceramide transporter (CERT/GPBP/StarD11) (6) are more distantly related to the StarD4 subfamily. Therefore, the StarD4 subfamily is likely to function in cholesterol metabolism. StarD4 and StarD5 are widely expressed, with highest levels in liver, whereas StarD6 expression is limited to testis. StarD4 was first identified using microarrays as a gene whose expression in mouse liver decreased ~3-fold upon cholesterol feeding (2). In both mouse liver and cultured cells, StarD4 showed the same pattern of gene regulation as known targets of the sterol regulatory element-binding proteins (SREBPs), whereas StarD5 was not sterol-regulated.

Two transcription factor families, the SREBPs and liver X receptors (LXRs), are implicated in gene regulation by cholesterol and its oxysterol metabolites (7). When sterols are low, SREBPs activate genes responsible for synthesis and uptake of cholesterol and fatty acids (8). Two SREBP genes encode three distinct proteins; SREBP-1a and SREBP-1c predominantly activate genes involved in fatty acid metabolism, whereas SREBP-2 targets are involved in cholesterol metabolism (8). When sterols are in excess, SREBPs are inactive, whereas the LXRs nuclear receptors are activated by oxysterol ligands. Most known LXR target genes function in reverse cholesterol transport, a process by which high density lipoproteins transport cholesterol from peripheral cells to the liver, where it is excreted in bile as free cholesterol or bile acids (9). There is cross-talk between LXRs and other transcriptional pathways, notably LXRs stimulating fatty acid synthesis by activating SREBP-1c expression (10). Recently, cholesterol was shown to activate a third transcriptional pathway, as free cholesterol-loaded macrophages trigger the endoplasmic reticulum (ER) stress response (11). Known ER stress target genes include ER chaperones, ER calcium channels, pro-apoptotic transcription domain containing 4–6; MLN64, protein of unknown function; PCTP, phosphatidylcholine transfer protein; SRE, sterol regulatory element; SREBP, SRE-binding protein; LXR, liver X receptor; LXRE, LXR element; ER, endoplasmic reticulum; HMG, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, HMG CoA reductase; HMGS, HMG CoA synthase; ORF, open reading frame; RT, reverse transcription; RACE, rapid amplification of cDNA ends; BiP, immunoglobulin binding protein.

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factors, and components of the secretory pathway (12), but additional targets could function in cholesterol metabolism.

In this study we address the differential gene regulation for StarD4 and StarD5. Although both showed STAR-like cholesterol transfer activity in a cell culture steroidogenesis assay, their expression is not activated like STAR during steroidogenesis. In the SREBP transgenic mouse model, StarD4 expression was preferentially induced by SREBP-2 compared to SREBP-1a. In cell culture reporter transfection assays, StarD4 and StarD5 showed two functional activities, 1) stimulation of steriodogenesis by the mitochondrial P450scc enzyme and 2) stimulation of LXR reporter activity. Because the known cholesterol transfer proteins STAR and MLN64 shared these activities, StarD4 and StarD5 likely also function in cholesterol metabolism.

MATERIALS AND METHODS

Animals and Diets—Transgenic mice expressing truncated nuclear forms of human SREBP-1a or SREBP-2 were obtained from Jackson Laboratory (catalog numbers 002840 and 003311) and backcrossed to C57BL/6. Mice were genotyped by PCR from tail tip DNA using primers in Supplemental Table I. N6/N7 generation SREBP-1a and N2/N3 SREBP-2 mice were used in these studies. Transgenic and littermate control male mice were fed standard rodent chow from birth to 8 weeks. They were then switched to a 65% protein, 10% carbohydrate diet beginning at 8 weeks of age. Male mice were genotyped by PCR from tail tip DNA using primers described previously (2). Mice on the SREBP-2 reporter strain were on the C57BL/6/129Sv background. Mice on the SREBP-1 reporter strain were on the C57BL/6 background. All genotypes were confirmed by PCR analysis of tail tip DNA (see Supplemental Table I). SREBP-1 and SREBP-2 transgenic mice were backcrossed to C57BL/6 mice to ensure that the SREBP promoters were under the control of the endogenous mouse SREBP promoters.

1a. In cell culture reporter transfection assays, luciferase reporter constructs were transiently transfected into MA-10 cells. MA-10 cells (a gift of Jonathan Smith) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. NIH-3T3 cells were grown as fibroblast-like cells and were not differentiated to adipocytes. Lipoprotein lipase (LPL) expression was measured by reverse transcription–PCR (RT-PCR) as previously described (2). The overall magnitude of the differences between genotypes was confirmed by northern blot analysis of poly(A)+ RNA from whole body tissues. Nucleotide sequences were PCR-amplified from C57BL/6 mouse liver or testis cDNA and cloned between SacI and HindIII tails into pGL3 basic vector (Promega). The entire SacI/HindIII fragment was subcloned into these sites in the pGL3 basic luciferase vector (Promega). To generate ~68 reporter constructs, BamHI (which cuts at ~68) and HindIII-digested promoter fragments were cloned into these sites of pGL3. The human StarD4 proximal promoter from ~521 to ~78 was similarly amplified from genomic DNA with BamHI and HindIII tails and cloned into pGL3.

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281 were generated to PCR amplify and re-clone these constructs. Briefly, total RNA was treated with phenol/chase, decapped, 5’-ligated to a RACE adaptor sequence, and reverse-transcribed. The resulting cDNA was the template for nested PCRs with 5’ inner and outer primers in the adaptor and 3’ gene-specific inner and outer primers. 5’ gene-specific primers served as controls, and the sequences of mouse and human StarD4 RACE primers are shown in Supplemental Table I. RACE inner PCR products were TA-cloned with pCR-2.1-TOPO (Invitrogen) and sequenced.

For anti-FLAG Western blots, whole cell lysates from transfected COS-1 cells were prepared in lysis buffer with protease inhibitor mixture (Sigma). Protein concentrations were measured using BCA reagent (Pierce), and 50 μg of protein was loaded in each well of pre-cast 12% polyacrylamide gels (Invitrogen) for SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Invitrogen), and blots were blocked in phosphate-buffered saline-casein (Pierce). The primary antibody was 1:500 anti-FLAG M2 (Sigma), and the secondary antibody was 1:2000 goat anti-mouse IgG horseradish peroxidase (Sigma). Blots were washed in phosphate-buffered saline-Tween and developed using Renaissance Chemiluminescence Reagent (PerkinElmer Life Sciences).

Rapid Amplification of cDNA Ends (RACE)—Total liver RNA was prepared from Chow-fed C57BL/6 mice, and pooled mouse total liver RNA was purchased from Clontech. The FirstChoice RLM-RACE kit (Invitrogen) was used according to manufacturer’s instructions. Briefly, total RNA was treated with phenol/chase, decapped, 5’-ligated to a RACE adaptor sequence, and reverse-transcribed. The resulting cDNA was the template for nested PCRs with 5’ inner and outer primers in the adaptor and 3’ gene-specific inner and outer primers. 5’ gene-specific primers served as controls, and the sequences of mouse and human StarD4 RACE primers are shown in Supplemental Table I. RACE inner PCR products were TA-cloned with pCR-2.1-TOPO (Invitrogen) and sequenced.

StarD4 Reporter Cloning and Mutagenesis—PCR primers were designed to amplify StarD4 promoter sequence from genomic DNA. The mouse StarD4 proximal promoter was amplified from C57BL/6 genomic DNA using a forward primer at ~1335 and reverse primers at either +34 or +157 (Supplemental Table I). The forward primer had a SacI and HindIII tails. The HindIII fragment was TA-cloned. To generate the ~1335 StarD4 reporters, the entire SacI/HindIII fragment was subcloned into these sites in the pGL3 basic luciferase vector (Promega). To generate ~874 reporters, BamHI (which cuts at ~874) and HindIII-digested promoter fragments were cloned into these sites of pGL3. To generate ~350 reporters, such as the 350/34 reporter used in most experiments, BamHI (which cuts at ~34) and HindIII fragments were cloned into these sites of pGL3. The human StarD4 proximal promoter from ~521 to ~78 was similarly amplified from genomic DNA with BamHI and HindIII tails and cloned into pGL3.

Mutations were generated in the mouse ~350/34 and human ~521/8 reporters using the GeneEditor in vitro site-directed mutagenesis system (Promega) according to manufacturer’s instructions. The absence of transgenic effects in these mice on the D1337 mutation indicates that site-directed mutations in the potential SREs and CCAAT boxes are shown in Supplemental Table I. The mutations were designed to add or remove restriction sites so mutant clones could be identified. All wild type and mutant StarD4 constructs were sequence-verified.

A potential SREBP-binding E box has been reported in the pGL3 proximal promoter between the SREBP-1a and SREBP-2 sites (17). To introduce the E box into the 350/34 reporter, the entire SacI/HindIII sites of pGL3. The human StarD4 proximal promoter from ~521 to ~78 was similarly amplified from genomic DNA with BamHI and HindIII tails and cloned into pGL3.

Cloning and PCR—All molecular cloning followed standard techniques using enzymes from New England Biolabs. PCR reagents were from Advanta cDNA polymerase kit (Clontech), primers were from Genelink, and thermal cycling was on a PerkinElmer Life Sciences 7700. All DNA constructs were sequence verified at the Rockefeller University DNA Sequencing Resource Center. DNA sequence was aligned and analyzed using the DNASTAR software package.

FLAG-STAR Expression Plasmids—STAR domain coding sequences were PCR-amplified from C57BL/6 mouse liver or testis cDNA using primers with restriction sites at their 5’ tails. The primers for StarD4 and StarD5 ORFs were as previously described (2), whereas the STAR START domain, MLN64 START domain, and PCTP ORF primer sequences are shown in Supplemental Table I. The PCR products were generally cloned into BamHI and XhoI sites of the pCMV-Tag2B FLAG epitope tagging mammalian expression vector (Stratagene). The mouse StarD5 sequence has an internal BamHI site, so it was cloned using 5’ PstI instead. Mouse STAR has an internal XhoI site but was cloned using this enzyme by partial digestion. All expression plasmids were sequence-verified using T3 and T7 primers to assure correct STAR domain sequence and in-frame fusion of the N-terminal FLAG epitope tag. Mouse StarD4 and PCTP had coding single nucleotide polymorphisms characteristic of the C57BL/6 strain (StarD4, Ala-121, Thr-152, PCTP, Gly-205, Gly-324, Gly-379), with those found in some other strains like FVB.

For anti-FLAG Western blots, whole cell lysates from transfected COS-1 cells were prepared in lysis buffer with protease inhibitor mixture (Sigma). Protein concentrations were measured using BCA reagent (Pierce), and 50 μg of protein was loaded in each well of pre-cast 12% polyacrylamide gels (Invitrogen) for SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Invitrogen), and blots were blocked in phosphate-buffered saline-casein (Pierce). The primary antibody was 1:500 anti-FLAG M2 (Sigma), and the secondary antibody was 1:2000 goat anti-mouse IgG horseradish peroxidase (Sigma). Blots were washed in phosphate-buffered saline-Tween and developed using Ren-
mg/ml for 22(R)-hydroxy-cholesterol, both in ethanol. To treat cells, these agents were added to media containing lipoprotein-depleted serum or fetal bovine serum at a final concentration of 1×, thus, 1:1000 of the stock concentrations above. Vehicle control experiments showed no effect of 0.1% ethanol or methanol in the various assays (data not shown). Treatments were preformed on subconfluent cells in 6-well plates for 18–20 h.

**Transient Transfection Experiments**—Before all transfection assays, cells were split into 24-well plates at 10^5 cells per well and grown overnight. The next day each well of 50–80% confluent cells was transfected with 200 ng of luciferase reporter and 20 ng of cytomegalovirus β-galactosidase expression vector for normalization. After 24–28 h, media was withdrawn, and progesterone was assayed by enzyme-linked immunosorbent assay (Diagnostic Biochem Canada). Data are presented as the mean and S.D. of triplicate wells.

For the steriodogenesis assay each well of COS-1 cells was transfected with 100 ng of F2 P450scc expression plasmid (a gift of Walter Miller, described in Ref. 18), 100 ng of 3β-hydroxysteroid reductase (a gift of Jerome Strauss, described in Ref. 19), and 100 ng of FLAG-START domain from StAR or MLN64 results in a stimulation of steriodogenesis (Fig. 1A). Although to a lesser extent, StarD4 and StarD5 also increase progesterone production, indicating STAR-like cholesterol transfer activity. As a negative control, the phosphatidylcholine binding START protein PCTP was inactive in this assay. Western blots against the FLAG epitope tag indicate that these START domains are overexpressed to similar levels, although StarD4 expression was consistently less (Fig. 1B).

**RESULTS**

**StarD4 and StarD5 in Steroidogenesis**—In adrenal and gonadal steroidogenic cells, STAR delivers cholesterol to the mitochondrial P450 side chain cleavage enzyme (P450scc) to generate the first steroid pregnenolone. This is converted to progesterone by 3β-hydroxysteroid reductase, and subsequent enzymatic steps generate other steroids. To assay for STAR-like activity, non-steroidogenic COS-1 cells can be transfected with P450scc and 3β-hydroxysteroid reductase to generate progesterone. As previously reported (24, 25), co-transfection of the START domain from STAR or MLN64 results in a stimulation of steriodogenesis (Fig. 1A). Although to a lesser extent, StarD4 and StarD5 also increase progesterone production, indicating STAR-like cholesterol transfer activity. As a negative control, the phosphatidylcholine binding START protein PCTP was inactive in this assay. Western blots against the FLAG epitope tag indicate that these START domains are overexpressed to similar levels, although StarD4 expression was consistently less (Fig. 1B).

**Data Analysis and Statistics**—For all quantitative assays, the control and experimental groups were compared by a two-tailed type 2 Student’s t test, with the threshold for significance at p < 0.05. Data were analyzed in Microsoft Excel and graphed in Prism (Graphpad). All bar graphs show the mean with S.D. error bars.

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Other Materials—Triplicate cDNA samples from control mouse macrophages and those loaded with free cholesterol or cholesterol esters were gifts of Bo Feng and Ira Tabas, described in Feng et al. (11). The assay for Xhp1 splicing and the mouse Xhp1 PCR primers were as described (23).
StarD4 expression is activated in SREBP-2 transgenic mouse liver. RNA was extracted from the livers of SREBP-1a and SREBP-2 transgenic male mice (Tg) as well as non-transgenic littermate controls (cont) (n = 5 per group). Expression of FABP5 (preferential SREBP-1 target), HMGS (preferential SREBP-2 target), and StarD4 was analyzed by quantitative RT-PCR normalized to β-actin. Gene expression in littermate controls for each transgene was set equal to one. *, p < 0.05 versus control.

expression in livers from these mice have previously been analyzed by Northern blots (26) or microarrays (27). Genes involved in cholesterol metabolism are activated by SREBP-2 and also SREBP-1a, but genes involved in fatty acid metabolism are highly regulated by SREBP-1a but not SREBP-2. Microarray analysis has shown StarD4 expression is induced 3.0–3.5-fold in SREBP-1a and 4.6–6.1-fold in SREBP-2 transgenic mice (27). We used quantitative PCR to analyze expression of StarD4 and control genes in similar SREBP-1a and SREBP-2 transgenic mice compared with non-transgenic littersates (Fig. 2). The control SREBP-1 target fatty acid-binding protein 5 (FABP5) and SREBP-2 target HMG CoA synthase (HMGS) showed the expected patterns of regulation (16). StarD4 expression was induced 5-fold in SREBP-2 transgenic mice (p < 0.01) but only 2-fold in SREBP-1a transgenic mice (p = NS). This pattern is consistent with SREBP-2 regulation, so StarD4 is a preferential SREBP-2 target likely to function in cholesterol metabolism.

Differences in the regulation of SREBP-1 versus SREBP-2 target genes have been reported in intact wild-type mice fed dietary cholesterol for 1 week. SREBP-2 target genes are immediately down-regulated after only 1 day of cholesterol feeding, whereas SREBP-1 targets take several days (16). When StarD4 expression was analyzed in such a time course of cholesterol feeding, its expression was fully decreased by ~66% after only 1 day of feeding (data not shown), consistent with other SREBP-2 target genes.

The StarD4 Promoter—StarD4 mRNA expression is sterol-regulated by SREBPs, an effect likely mediated by a promoter SRE. To locate the StarD4 promoter by defining the 5′ mRNA end, 5′-RACE was performed on mouse liver RNA. Sequencing 25 RACE products revealed eight initiation sites 104–149 bp upstream of the ATG codon, with the most common being A-137 (10/25) and A-107 (8/25) (Fig. 3A). A-137 was defined as the +1 site for numbering the mouse promoter. The human StarD4 gene differs from the mouse gene, with its ATG start codon located in the second exon, resulting in a protein 16 amino acids shorter at the N terminus (2). 5′ RACE analysis of human liver StarD4 also revealed multiple initiation sites, with the most common G-131 from the 3′ end of exon 1 (8/12 clones) and defined as +1. There is no apparent TATA box in either StarD4 promoter, consistent with multiple initiation sites in TATA-less genes (28). Several other SREBP targets have TATA-less promoters and multiple initiation sites, including HMGR (29) and 7-dehydrocholesterol reductase (30).

Mouse StarD4 sequence from −350 to −100 is 68% identical to the sequence from −281 to −10 in the human promoter (Fig. 3A). This is very high sequence conservation for non-coding DNA, indicating the presence of conserved gene regulatory elements. There are three conserved CCAAT boxes and two GC boxes, potential binding sites for NF-Y and Sp1, respectively. Based on the rough SRE consensus YCAANYCAY (see “Discussion”), five potential SREs were identified in the mouse promoter at −300, −279, −239, −199, and −132 and designated A–E. The proximal three of these, mouse potential SREs A–C, align with corresponding potential SREs in the human promoter at −221, −200, and −159. In addition to the StarD4 proximal promoter, a more distal region was also conserved at −1221–−1110 in mouse and −1898–−1793 in human StarD4 (Fig. 3B). In this stretch of ~100 bp there was >80% interspecies nucleotide identity, although no potential SREs were apparent. This conserved upstream element may represent another regulatory region.

StarD4 Reporter Transfection Studies—To identify a functional SRE, luciferase reporters driven by the StarD4 promoter were cloned and transfected into NIH-3T3 cells. Mouse StarD4 promoter −350/+34 reporter activity decreased 9-fold upon culture in cholesterol and 25-hydroxycholesterol, which repress SREBP activity, but increased 3-fold upon culture in lovastatin, which depletes cellular cholesterol and activates SREBPs (Fig. 4A). The positive control SREx3 reporter was similarly regulated, whereas the negative control pGL3 vector was unregulated. Furthermore, the mouse StarD4 reporter showed dose-dependent repression in response to 25-hydroxycholesterol and dose-dependent activation in response to lovastatin (data not shown). The −350/+34 mouse reporter contains all elements necessary for sterol regulation, as it showed similar activity and sterol regulation to four longer reporters: −350/−137, −1335/−137, −1335/+34, and −874/+34 (data not shown). The two constructs spanning to −1335 included the putative enhancer element but failed to show higher activity than shorter −350 constructs with only the proximal promoter. The human StarD4 reporter was regulated similarly to the mouse reporter, although its fold regulation was consistently less (only ~10-fold rather than ~20-fold comparing sterols to lovastatin, see Fig. 5). Two different lengths of human promoter (−521/−8 and −282/−8) reporters also showed no differences in activity or regulation (data not shown).

By site-directed mutagenesis, point mutations were introduced into the three conserved SREs of the mouse and human StarD4 reporters (see Fig. 3A). SRE-A or SRE-C mutations had little effect on -fold sterol regulation, whereas SRE-B mutations markedly decreased sterol regulation in both species (Fig. 4, B–C). Additional experiments showed that mutations in potential mouse SRE-D or SRE-E also did not affect sterol regulation (data not shown).

Because SRE-B appears to be the functional element, we studied SRE-B mutant reporters in more detail. When SREBPs were repressed by sterols, wild type and SRE-B mutant reporters had the same absolute activities (Fig. 5, A–B). However, when cells were cultured in the control media (basal SREBP activity) or in lovastatin (activated SREBPs), the wild type reporters were strongly activated, whereas the SRE-B mutant reporters were only weakly activated. Therefore, most of the sterol regulation of mouse and human StarD4 appears mediated by SRE-B.

Mutations were also generated in two CCAAT boxes near SRE-B (see Fig. 3A), since in other SRE-containing promoters these elements bind NF-Y and cooperate with SREBPs to ac-
Fig. 3. Alignment of mouse and human StarD4 promoters. A, the StarD4 proximal promoters. Bases conserved between species are indicated with asterisks, whereas gaps are indicated by dashes. Boxes surround potential CCAAT boxes (double lines), GC boxes, SREs, and ATG start codons in both genes. The minimal mouse luciferase reporter spans −350 to +34, whereas the minimal human reporter spans from −281 to −8 (< or > symbols). Site-directed mutations of elements introduced into reporters are shown in italics above or below the sequences. Transcription initiation sites identified by RACE are boxed along with the number of clones (out of 25 total in mouse and 12 total in human). Also boxed is an in-frame TGA stop codon in exon 1 of the mouse gene. The StarD4 coding sequence (amino acid sequence shown) begins in exon 1 for mouse but in exon 2 for human. The intervening intron 1 (not shown, ellipse) is 4476 bp in mouse and 4942 bp in human, and both genes are abbreviated within exon 2 in this figure. B, a more distant upstream element conserved between mouse and human StarD4.

Fig. 4. Regulation of StarD4 luciferase reporters by sterol and statin treatment. Triplicate wells of NIH-3T3 cells were transiently transfected with luciferase reporters and a β-galactosidase expression plasmid for normalization. They were then cultured in lipoprotein-deficient media with sterols or statin for 18–20 h (see “Materials and Methods”). A, the mouse StarD4 reporter was regulated like the positive control SRE-regulated reporter (SREx3), whereas the empty luciferase vector (pGL3) was unregulated. Control activity for each reporter was set equal to one. *, p < 0.05 versus control. B–C, site-directed mutations were generated in SRE-A, SRE-B, or SRE-C of mouse −350/+34 and human −521/−5 StarD4 reporters. Sterol-treated activity for each reporter was set equal to one. *, p < 0.05, mutant versus wild type (statin-treated).

activate transcription (31). When the CCAAT box +10 from SRE-B was mutated, the mouse reporter was virtually unregulated, with low activity even in the presence of lovastatin (Fig. 5C). Mutations in the CCAAT box +40 from SRE-B also blunted sterol regulation. Similar results were obtained with both corresponding CCAAT box mutations in the human StarD4 reporter (data not shown). It appears that both of these CCAAT boxes, in addition to SRE-B, are necessary for maximally activated transcription from the StarD4 promoter.

StarD4 and StarD5 Effects on LXR—In addition to affecting SREBP s, cholesterol can also regulate gene expression via LXRs, so we assayed for LXR regulation of StarD4 and StarD5. When mice were treated with the synthetic LXR agonist T0901317, liver expression of the known LXR-target gene ABCG5 was induced almost 3-fold, whereas StarD4 and StarD5 mRNA levels were unchanged (Fig. 6A). These mice developed the expected hepatic steatosis (apparent by visual inspection), indicating LXR activation of SREBP-1c and fatty acid synthesis (32, 33). Because StarD4 was not induced, it appears to be a poor target for SREBP-1 in mouse liver, consistent with data from the SREBP-1a transgenic mice (see Fig. 2).

We also treated RAW mouse macrophage cells with LXR ligands. As expected, ABCA1 mRNA levels were highly regulated (34), 6-fold by the weak LXR agonist 25-hydroxycholesterol and 30-fold by the strong agonist T0901317 (Fig. 6B). Expression of StarD4 was decreased 4-fold by 25-hydroxycholesterol, consistent with its known sterol regulation via SREBPs (2). The LXR agonist caused a 2-fold increase in StarD4, but this regulation was also observed for another SREBP-2 target, HMGS. Conversely, StarD5 was unregulated by oxysterol or synthetic LXR ligands in RAW cells. Similar results for StarD4 and StarD5 were observed in Hepa-1 hepatoma cells (data not shown). Therefore, StarD5 is not an LXR target, whereas weak LXR activation of StarD4 is consistent with SREBP-1c effects.

Even if they are not LXR target genes, StarD4 or StarD5 expression could affect transcriptional activation by LXRs. COS-1 cells were transfected with an LXHR luciferase reporter, expression plasmids for human LXRα and retinoid X receptor α, and FLAG-START expression plasmid. The StAR and MLN64 START domains both stimulated LXRE activity 5.5-fold, similar to the effects of the synthetic LXR ligand T0901317 (Fig. 6C). StarD4 and StarD5 likewise activated the LXRE reporter 3–4-fold, whereas PCTP had no effect. These START domains could activate LXRs as well as LXRo (data not shown). Therefore, even though StarD4 and StarD5 are not themselves transcriptional targets of LXRs, their overexpression can stimulate LXR activity.

ER Stress Activation of StarD5 Expression—In addition to SREBPS and LXRs, a third transcriptional pathway can be regulated by cholesterol. It was recently shown that free cholesterol-loaded macrophages activate various components of the ER stress response (11). To confirm these findings, we used a previously described RT-PCR assay for splicing of the Xbp1 mRNA (23), an effect mediated by the endonuclease activity of IRE-1 in response to ER stress. Processed Xbp1 appeared in
The mouse and human StarD4 promoters were studied in transient transfection reporter assays. Two regions of upstream sequence were highly conserved, the proximal promoter and a more distal element. Upon transfection into NIH-3T3 cells, luciferase reporters driven by the mouse or human StarD4 promoter were sterol-regulated, as predicted by the regulation of endogenous StarD4 mRNA (2). Activity and regulation were observed for proximal promoter constructs, and the distal element had no discernable effect. Each potential SRE in the mouse and human StarD4 promoters was mutated, and only mutations in one had any effect. Sterol regulation was reduced by a factor of 4–5 upon mutating SRE-B in either mouse or human StarD4 promoters, data not shown). These results indicate StarD5 expression is specifically induced by the ER stress response.

**Discussion**

StarD4 was originally identified as a gene repressed by dietary cholesterol in mouse liver, whereas StarD5 was identified by homology to StarD4. The current study further characterizes StarD4 and StarD5 in three ways. First, the mechanism of sterol regulation of StarD4 is described, as StarD4 is an SREBP-2 target gene with a conserved functional SRE in its proximal promoter. Second, StarD5 is not regulated by the SREBP or LXR transcription factors, but its expression is induced by the ER stress response. Third, StarD4 and StarD5 showed functional activity in two different transient transfection overexpression assays. SREBP-like activity in a steroidogenesis assay indicated cholesterol transfer, whereas activation of LXR transcriptional activity may reflect oxysterol generation.

Results in three mouse models indicate StarD4 is a preferential target for SREBP-2 versus SREBP-1a or -1c. 1) In SREBP transgenic mice StarD4 was more highly induced by SREBP-2 than SREBP-1a, 2) in wild-type mice fed a high cholesterol diet, StarD4 expression was maximally decreased after only 1 day of feeding (see Ref. 16), and 3) mice treated with 1X agonist activated SREBP-1c without affecting StarD4 expression. StarD4 expression was also coordinately activated with known SREBP-2 targets in two cell culture models, 1) MA-10 Leydig cells treated with a cAMP analog activated steroid synthesis from cholesterol and increased expression of StarD4 and HMGR, and 2) RAW macrophages treated with LXR agonist weakly increased expression of StarD4 and HMGS, an effect likely mediated by SREBP-1c. Therefore, although StarD4 can be activated by SREBP-1, it is a preferential target for SREBP-2.

The mouse and human StarD4 promoters were studied in transient transfection reporter assays. Two regions of upstream sequence were highly conserved, the proximal promoter and a more distal element. Upon transfection into NIH-3T3 cells, luciferase reporters driven by the mouse or human StarD5 promoter were sterol-regulated, as predicted by the regulation of endogenous StarD5 mRNA (2). Activity and regulation were observed for proximal promoter constructs, and the distal element had no discernable effect. Each potential SRE in the mouse and human StarD5 promoters was mutated, and only mutations in one had any effect. Sterol regulation was reduced by a factor of 4–5 upon mutating SRE-B in either mouse (20- to 4-fold) or human (12- to 3-fold). Interspecies conservation of SREs has been previously described for human and hamster HMGS (35). There was residual low sterol regulation of SRE-B mutant StarD4 reporters, particularly when lovastatin treatment maximally activated SREBPs. This may be mediated by one of the other potential SREs, although studies of SRE-B double mutant StarD4 reporters failed to clearly demonstrate this (data not shown). It is also unclear why StarD4 is a preferential target for SREBP-2 rather than StarD5.
SREBP-1, but this is a major unresolved issue for all SREBP targets.

Functional SREs with considerable sequence variation have been identified in many promoters (31). Nonetheless, the rough 9-bp consensus YCA(Y/C)/A/G/YCA(Y = pyrimidine) (36) is useful. Putative SREs resembling the consensus often bind SREBPs in electrophoretic mobility shift assays despite the fact that mutation has no effect on reporter regulation (31). Although there are five potential SREs in the mouse StarD4 promoter, only SRE-B affected sterol regulation. Non-functional SRE-A (CCACGCCAC) was the best candidate, since it was identical in mouse and human and differed from consensus only at position 4 (underlined). SRE-B in mouse (TCGGTCAT) was a weak candidate with three divergent bases, whereas human SRE-B (TCATTCAT) had better agreement.

All these differences are found in other SREs; G at position 3 in hamster HMGR (37), G at position 4 in fatty acid synthase and others (36), and T at the position 5 in mouse acetyl CoA synthase I (38). Despite two nucleotide differences between mouse and human StarD4 SRE-B, these elements were functionally conserved. These findings underscore the divergence in SRE sequence, the difficulty in identifying SREs by sequence alone, and the necessity of mutated promoter-reporter constructs to define functional elements.

The activity of mouse StarD4 SRE-B, despite relatively poor consensus agreement, likely reflects its position relative to other sites. All known SRE-containing promoters have a site for NF-Y (CCAT box) or Sp1 (GC box) in close proximity, as SREBPs interact with these factors to activate transcription (39). In promoters with cooperative GC boxes, this element lies within several nucleotides of the SRE. Therefore, the StarD4 GC box, >100 bp from SRE-B, is unlikely to cooperate. However, the StarD4 promoter has two conserved CCAAT boxes at +10 and +40 from the SRE-B, and mutations in either CCAAT box nearly abolished SREBP activation. Therefore, both CCAAT boxes and SRE-B are necessary for activated transcription from the StarD4 promoter. In other promoters spacing of the CCAAT box from the SRE appears crucial (31). Sterol regulation is abrogated upon increasing the spacing beyond 17 bp in the HMGS promoter (40) or beyond 20 bp in the farnesyl diphosphate synthase promoter (41), and the optimal spacing for the SREBP-2 promoter was 16–20 bp (35). However, in the stearoyl-CoA desaturase-1 and -2 genes, CCAAT elements 5 and 48 bp from the SRE were functional (42). Therefore, spacing may be promoter-specific, and CCAAT elements ranging from +5 to +50 from the SRE may be required for maximal activation.

StarD5 shows a similar tissue expression pattern to StarD4, and the proteins share 30% identity (2). However, StarD5 is not

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**Fig. 6.** StarD4 and StarD5 are not LXR target genes, but START domain expression can stimulate LXR activity. A, mice were treated by gavage with synthetic LXR agonist T0901317 or vehicle control (n = 5 per group) for 2 days, and liver RNA was extracted. Gene expression for ABCG5 (hepatic LXR target), StarD4, and StarD5 were assayed by quantitative RT-PCR normalized to cyclophilin. B, RAW mouse macrophage cells were treated for 18–20 h with either 25-hydroxycholesterol or T0901317 (n = 3 per group) before RNA extraction. Gene expression was assayed as above for StarD4, StarD5, ABCA1 (an LXR target), and HMGS (an SREBP-2 target). C, COS-1 cells were co-transfected with an LXR-regulated luciferase reporter (LXRE Luc) and expression plasmids for LXRA and retinoid X receptor α as well as the indicated FLAG-tagged START expression plasmid. Luciferase activity was normalized to β-galactosidase, and activity of the control empty FLAG vector was set equal to one. As a positive control, adding the synthetic LXR agonist T0907317 (agonist) activated LXR activity as expected. Activity was also stimulated by four START domains, but not PCTP. *, p < 0.05 versus control.
an SREBP target as its mRNA expression was unregulated by cholesteryl feeding in mice, by oxysterols or lovastatin in cultured cells (2), or by truncated nuclear SREBPs in transgenic mice (data not shown). StarD5 expression was also unregulated in MA-10 Leydig cells upon stimulation of steroidogenesis, which highly activates the STAR family prototype StAR and weakly activates SREBP-2 targets like StarD4 and HMGR. StarD5 expression was also unaffected by LXR agonists in mouse liver and cultured cells. However, StarD5 mRNA was increased 4-fold in free cholesterol-loaded macrophages. Tabas and co-workers have used Western blots to show activation of increased 4-fold in free cholesterol-loaded macrophages. Tabas and co-workers have used Western blots to show activation of increased 4-fold in free cholesterol-loaded macrophages. Because control ER stress reporters and the endogenous StarD5 mRNA were robustly induced in the same NIH-3T3 cells, the responsible elements must lie elsewhere in the gene. ER stress response elements have only been described in proximal promoters, but there are only a limited number of known target genes (43).

To address potential functions of StarD4 and StarD5, their subcellular location is a crucial issue. StarD4 and StarD5, like PCTP, consist entirely of START domains and lack apparent localization signals (2). Full-length StarD has an N-terminal signal that mediates its mitochondrial import, whereas full-length MLN64 has an N-terminal MENTAL transmembrane domain that localizes it to late endosomal membranes (1). However, the truncated isolated START domains of StAR (44) and MLN64 (45) localize diffusely throughout the cell cytoplasm and nucleus. Our studies used similar START-only StAR and MLN64 constructs along with full-length PCTP, StarD4, and StarD5, all mouse proteins with N-terminal FLAG tags. Upon transfection into HEK-293 cells and anti-FLAG immunofluorescence, START-only StAR and MLN64 and full-length PCTP showed the expected diffuse localization throughout cells. Localization of StarD4 and StarD5 was indistinguishable from the other three START domains, without any suggestion of punctuate staining to indicate a specific subcellular compartment (data not shown). Although these studies do not rule out specific localization of endogenous proteins at normal expression levels or regulated relocation in response to stimuli, it appears StarD4 and StarD5 are cytosolic proteins.

StarD4 and StarD5 showed activity in two cell culture assays, StAR-like activation of steroidogenesis by P450cc and activation of an LXR reporter. The cholesterol binding START domains of StAR and MLN64 were active in both assays, whereas PCTP was an inactive negative control. Although it appears that StarD4 and StarD5 were less active than StAR and MLN64 in both assays, relative activities remain uncertain given the differences in protein expression levels. STAR-like steroidogenic activity reflects delivery of cholesterol substrate to the inner mitochondrial membrane, where the P450cc enzyme resides (3). Although StarD4 and StarD5 are unlikely to function physiologically in steroidogenesis, their STAR-like activity in this assay is strong evidence that they are also cholesterol transport proteins.

Sterol 27-hydroxylase (Cyp27) is another cholesterol-metabolizing P450 enzyme on the inner mitochondrial membrane (46). In contrast to P450cc, Cyp27 is expressed in liver, macrophages, and many other cell types. Others have shown that StAR can deliver cholesterol substrate to Cyp27 (47). It is likely that the MLN64 START domain, StarD4, and StarD5 share this activity with StAR, and generation of the LXR agonist 27-hydroxycholesterol (48) would account for their stimulation of LXR reporter activity. In liver, 27-hydroxycholesterol is also the first intermediate in alternative bile acid synthesis, and StAR overexpression in primary hepatocytes stimulates this pathway (49). Because StAR is not expressed in liver and MLN64 is localized to late endosomal membranes, StarD4 or StarD5 may stimulate alternative bile acid synthesis in vivo. In macrophages, which are key in the development of atherosclerotic lesions, 27-hydroxycholesterol is implicated in LXR activation and cholesterol efflux (50, 51).

Other models not involving mitochondrial Cyp27 may also account for the observed LXR activation by START domains. The active START domains could deliver substrate to cholesterol 25-hydroxylase in ER and Golgi, and 25-hydroxycholesterol activates LXR agonists (48). Effects on cholesterol synthesis could also account for LXR activation, as 24(S),25-epoxycholesterol derived from the synthetic pathway is a high affinity LXR ligand (52). Finally, it is possible that the START domains have...
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direct transcriptional effects as LXRs coactivators, but four different START domains showed activity arguing against specific coactivator activity. Given the extensive literature that STAR delivers cholesterol to mitochondria, it is likely that these START domains deliver cholesterol to Cyp27 as opposed to alternate models.

Several hypotheses can now be generated about StarD4 and StarD5 function given their gene regulation patterns and sterol transfer activity. Because other known SREBP-2 target genes function in cholesterol synthesis or uptake (31), we propose four roles StarD4 could play in these pathways. First, StarD4 could act in cholesterol synthesis by transferring hydrophobic cholesterol precursor sterols. Second, StarD4 could facilitate movement of nascent cholesterol from the ER to the plasma membrane, a non-vesicular transport pathway (22) that may be considered the final step of cholesterol synthesis. Third, cholesterol taken up via low density lipoprotein receptor must leave cells more easily than cholesterol. Further experiments confirm that StarD5 could deliver cholesterol for esterification by the ER-localized enzyme acyl-coenzyme A:cholesterol acyltransferase (23), thus reducing free cholesterol levels. Third, StarD5 may generate oxysterols, which would negatively feedback on oxysterol synthesis and promoting cholesterol efflux.

Genes activated by ER stress like StarD5 either aid in restoring normal ER function or trigger apoptosis if ER function cannot be rescued. The ER is a key compartment in cholesterol metabolism (22), so StarD5 may play a protective role in ER-stressed cells. Because excess ER free cholesterol causes ER stress (11), we propose four models whereby StarD5 could reduce ER free cholesterol levels. First, StarD5 could transport cholesterol from the ER to other compartments or to cellular efflux pathways, perhaps involving ABC transporters. Second, StarD5 could deliver cholesterol for esterification by the ER-localized enzyme acyl-coenzyme A:cholesterol acyltransferase, thus reducing free cholesterol levels. Third, StarD5 may generate oxysterols for LXR activation of genes involved in cellular cholesterol efflux. Fourth, oxysterol generation itself may be a means to unload cholesterol, as more hydrophilic oxysterols leave cells more easily than cholesterol. Further experiments will be necessary to distinguish among these hypotheses for StarD4 and StarD5 function, as these genes may play important roles in intracellular cholesterol metabolism and the development of atherosclerosis.

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