Human StarD5, a cytosolic StAR-related lipid binding protein

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Abstract **Recently identified StarD5 belongs to the StarD4 subfamily, a subfamily of steroidogenic acute regulatory related lipid transfer (START) domain proteins that includes StarD4 and StarD6, proteins whose functions remain unknown. The objective of this study was to confirm StarD5's protein localization and sterol binding capabilities as measures to pursue function. Using rabbit polyclonal antibody against newly purified human histidine-tagged/StarD5 protein, StarD5 was detected in human liver. In parallel studies, increased expression of StarD5 in primary hepatocytes led to a marked increase in microsomal free cholesterol. Cell fractionation studies demonstrated StarD5 protein in liver cytosolic fractions only, suggesting StarD5 as a directional cytosolic sterol carrier. Supportive in vitro binding assays demonstrated a concentration-dependent binding of cholesterol by StarD5 similar to that of the cholesterol binding START domain protein StarD1. In contrast to selective cholesterol binding by StarD1, StarD5 bound the potent regulatory oxysterol, 25-hydroxycholesterol, in a concentration-dependent manner. StarD5 binding appeared selective for cholesterol and 25-hydroxycholesterol, as no binding was observed for other tested sterols. The ability of StarD5 to bind not only cholesterol but also 25-hydroxycholesterol, a potent inflammatory mediator and regulatory oxysterol, raises basic fundamental questions about StarD5's role in the maintenance of cellular cholesterol homeostasis.**—Rodriguez-Agudo, D., S. Ren, P. B. Hylemon, K. Redford, R. Natarajan, A. Del Castillo, G. Gil, and W. M. Pandak. **Human StarD5, a cytosolic StAR-related lipid binding protein.** ***J. Lipid Res.*** **2005.** 46: **1615–1623.**

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Cholesterol, a structural component of mammalian cell membranes, also serves as a precursor to bile acids and steroid hormones. Homeostasis of cholesterol within the body is maintained through the coordinated regulation of its cell-mediated uptake, transport/trafficking, sorting, biosynthesis, storage (i.e., esterification), secretion, and degradation to bile acids (1). More specifically, the steroidogenic acute regulatory related lipid transfer (START) domain superfamily of proteins has been shown to be involved in several pathways of intracellular trafficking of cholesterol (2, 3). It has been predicted that all proteins with a START domain contain a similar binding pocket, with modifications in that pocket determining ligand binding specificity and function (4). The START domains appear in a wide range of proteins and have been implicated in several cellular functions, including lipid transport and metabolism, signal transduction, and transcriptional regulation (3, 5). The recently discovered START-related lipid transfer protein StarD5 belongs to the StarD4 subfamily, a START subfamily containing StarD4, StarD5, and StarD6. The StarD4 subfamily has been shown to contain 205–233 amino acid residues, sharing 26–32% identity with each other (6). The nearest related proteins to the StarD4 subfamily are StarD1 and MLN64/StarD3, both of which have been shown in vitro to bind cholesterol at a stoichiometry of 1:1 (7) and, with increasing expression, to stimulate steroidogenesis (8–10). Recently, it was reported that overexpression of StarD4 and StarD5 could increase steroidogenesis in an in vitro assay, evidence for their ability to transfer cholesterol (11).

The first START domain protein crystal structure reported was that of the C-terminal portion of human MLN64/StarD3 (7), followed by the structure of mouse StarD4 and PCTP/StarD2 (12, 13). Both MLN64/StarD3 and StarD4 protein structures revealed similar secondary structural elements and a hydrophobic tunnel with a size consistent with the binding of one cholesterol molecule (7, 12). Less related to the StarD4 subfamily but also containing a START domain with a known lipid ligand is the phosphatidylcholine transfer protein PCTP/StarD2 (14). The crystal structure of PCTP/StarD2 also shows a hydrophobic

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tunnel, but in contrast to MLN64/StarD3 and StarD4, it appears to selectively bind phosphatidylcholine (13).

Although the roles of proteins such as StarD1, PCTP/StarD2, and MLN64/StarD3 have been studied extensively with a better appreciation of their possible roles (8–10, 12–16), the role(s) of the proteins of the StarD4 subfamily remain unclear. In contrast to StarD1 and MLN64/StarD3, the proteins StarD4, StarD5, and StarD6 do not have N-terminal targeting sequences that could direct these proteins to specific cellular organelles. Therefore, they are predicted to be cytoplasmic proteins, like PCTP/StarD2 (6, 17). Furthermore, it remains unclear whether this StarD4 subfamily of proteins is capable of binding cholesterol and/or other sterols.

The expression of the StarD4 subfamily of proteins within different tissues has been shown at the mRNA level only. StarD4 and StarD5 were shown to be expressed in several mouse tissues, but they are most abundant in the liver and kidney. In contrast, StarD6 appears in mouse testis but not in the ovary, suggesting a specific role for StarD6 related to fertility (6). With respect to the regulation of these proteins, there are only relatively preliminary data about StarD4. Gene expression of StarD4 has been shown to be downregulated by cholesterol, as demonstrated by microarray analysis of cholesterol-fed mouse liver (6). These findings have since been corroborated through microarray analysis in transgenic mouse models, in which the transcriptional rates of StarD4 were found to be increased in the livers of transgenic mice overexpressing sterol response element binding protein (SREBP) isoforms. Conversely, in mice lacking all nuclear SREBPs, as mediated through gene knockout of SREBP cleavage-activating protein, liver StarD4 expression was lower. These studies show that the StarD4 gene is a SREBP target gene (15). Although present, the regulation of StarD5 by sterols appears much more modest and will require additional study (6).

The objective of this study was to purify human StarD5 and to confirm its cellular localization and sterol binding capabilities as initial measures to further pursue this novel protein's function. Using a newly generated polyclonal antibody to human StarD5, this study demonstrates the detection of StarD5 protein within the liver. Furthermore, in vitro binding assays demonstrating StarD5's ability to bind both cholesterol and 25-hydroxycholesterol, coupled with the observation of increased intracellular cholesterol accumulation with increasing StarD5 expression, are supportive of StarD5 as a cytosolic sterol carrier.

EXPERIMENTAL PROCEDURES

Materials

The following materials were purchased from Novagen: His-Bind Resin, 8× charge buffer, and 4× strip buffer. For cholesterol binding assays, a nickel-nitrilotriacetic acid agarose resin from Qiagen was used. Antibiotics for bacterial cultures were purchased from Sigma. Enzymes used for cloning procedures were purchased from Promega. The labeled sterols [^{14}C]cholesterol and [^{3}H]25-hydroxycholesterol were purchased from Perkin-Elmer; [^{14}C]27-hydroxycholesterol was made in our laboratory (see Generation of [^{14}C]27-hydroxycholesterol below), and 7α-hydroxycholesterol, 24-hydroxycholesterol, 24,25-hydroxycholesterol were obtained from Steraloids. Cholic acid was obtained from Steraloids and β-sitosterol from Sigma.

Bacterial strains and vector

The *Escherichia coli* strain Novablue (rec*A*, end*A*, lac*I*^{q}) was used for the construction of clones. For protein expression, the *E. coli* strain BL21(DE3) with the cDNA cloned into the inducible vector pET-24b(+) was used.

Oligonucleotide synthesis

All oligonucleotides were synthesized at the Sigma-Genosys facility. The oligonucleotides were resuspended in water to a final concentration of 100 μM.

General cloning procedures

Digestion with restriction enzymes and ligations were carried out according to the manufacturer's instructions (Promega), and transformation was carried out by calcium chloride. Growth of bacteria and nucleic acid electrophoresis were performed by standard procedures as described by Ausubel et al. (18).

Vector and PCR product isolation and purification

Plasmids were purified from *E. coli* strains by an alkaline lysis method (18) using the QIAprep-Spin Miniprep Kit (Qiagen). DNA fragments obtained by PCR were purified using the QIAquick PCR Purification Kit (Qiagen). Plasmids and PCR fragments, digested with restriction enzymes, were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen).

PCR procedure

PCR experiments were carried out in a total volume of 0.1 ml. PCR performed with the StarD5 cDNA contained PCR buffer (1×), 1.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 20 ng of template, 1 μM of each primer, and 1 unit of *Taq* polymerase. Amplifications were performed using the following reaction conditions: 1 cycle of 2 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. The reaction was finished with one cycle of 10 min at 72°C. The PCR products were then analyzed by agarose gel electrophoresis.

Cloning strategy

The N-terminal truncated StarD1 protein, (N62)StarD1, containing the *Nde*I and *Xho*I restriction sites, was obtained by PCR using the plasmid pET-24b(+)/hStarD1 as template and the following primers: 5′-GGGAATTCCATATGCTGGAAGAGACTCTC-3′ and 5′-CCCGGCCTCGAGACACCTGGCTTCAGAGGCC-3′ (*Nde*I and *Xho*I restriction sites are underlined). The PCR product was purified and then digested with enzymes *Nde*I and *Xho*I. After digestion, the fragment was ligated into pET-24b(+) vector that was previously digested with *Nde*I and *Xho*I and purified, creating a C-His-tag/(N62)StarD1 DNA. The ligation mixture was used to transform Novablue competent cells. The single colonies grew on LB agar plates and were screened for the 669 nucleotide fragment by digestion with *Nde*I and *Xho*I, corresponding to the (N62)StarD1 coding region. Following the same strategy, the StarD5 DNA was obtained by PCR using the plasmid pZEROTG-CMV/hStAR-D5 as template and the primers 5′-ACTTTATACCATATGGACCCG-GCGCTGGCAGCC-3′ and 5′-GCGATAGTACTCGAGCTCATG-GAATTGCTTCACTGCTTT-3′ (*Nde*I and *Xho*I restriction sites are underlined) and cloned into pET-24a(+) vector. For further confirmation, the plasmids with the (N62)StarD1 and StarD5 coding regions were sequenced. Finally, the plasmids were used to transform BL21(DE3) competent cells for protein expression.

Purification of (N62)StarD1 and StarD5 proteins

To purify His-tag/StarD5 and His-tag/StarD1, BL21(DE3) cells containing pET-24b(+)/(N62)StarD1 or pET-24b(+)/StarD5 plasmids were grown in 10 ml of LB broth with 100 μg/ml kanamycin at 37°C overnight. Then, 6.25 ml of this culture was used to inoculate 1 liter of LB broth containing 0.36% glucose. The 1 liter culture was grown at 37°C with shaking until the optical density at 600 nm reached 0.3–0.6 (~3 h). At this point, 1 ml of a 1 M isopropylthio-β-galactoside solution was added and incubated for an additional 4 h at 37°C with shaking. Cells were harvested by centrifugation (8,000 rpm for 10 min at 4°C), resuspended in 300 mM NaCl, 50 mM NaH_2PO_4, and 20 mM Tris, pH 7.4 (lysis buffer), and frozen at −20°C. The next day, the extracts were thawed on ice and sonicated. At this point, PMSF was added to the solution at a final concentration of 0.5 mM. Bacterial lysates were centrifuged at 14,000 rpm for 20 min at 4°C. The His-Bind resin was prepared according to the manufacturer's instructions and equilibrated with lysis buffer. The supernatant was incubated with His-Bind resin for 30 min in the column at 4°C. The column was washed with 2 volumes of lysis buffer (wash buffer I) and then with lysis buffer plus 60 mM imidazole (wash buffer II) until the absorbance at 280 nm was <0.01. His-tag/proteins were eluted using 4 volumes of lysis buffer plus 1 M imidazole, and 1 ml fractions were collected. Protein from each step of the His-tag purification method was analyzed by SDS-PAGE under reducing conditions on a 12% gel, according to the method described by Laemmli (19).

Protein sequencing via Edman chemistry

For N-terminal sequencing, 3 μg of pure protein was immobilized onto a polyvinylidene difluoride membrane as described below but performing the electroblot procedure with 10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11, and 10% methanol. The membrane was then stained with 0.1% Coomassie solution for 60 s and immediately destained with 50% methanol with constant shaking. Finally, the membrane was rinsed with water and air dried for 60 min. The protein band of interest was cut out and sent to the Molecular Structure Facility of the University of California (Davis, CA) for N-terminal sequencing.

Western blot analysis for (N62)StarD1 and StarD5

Total protein samples from (N62)StarD1 and StarD5 expression and purification were analyzed by immunodetection using His-tag antibodies (Sigma). Samples were separated on 12% SDS-PAGE gels and transferred onto a polyvinylidene difluoride membrane using a Bio-Rad Mini-Blot transfer apparatus (Bio-Rad, Hercules, CA) at 100 V for 1 h. The membrane was transferred to a blocking solution, 10% nonfat dry milk in PBS-wash buffer (1.7 mM NaH_2PO_4, 8 mM Na_2HPO_4, 145 mM NaCl, and 0.1% Tween 20) at 4°C overnight with shaking. The membrane was then incubated in 2% nonfat dry milk in PBS-wash buffer containing a 1:3,000 dilution of a primary antibody (Monoclonal Anti-Polyhistidine; Sigma) at room temperature for 1 h with shaking. The membrane was then washed three times in PBS-wash buffer, 10 min/wash at room temperature. After washing, the membrane was incubated in a 1:4,000 dilution of a secondary antibody (goat anti-mouse IgG-HRP conjugate; Bio-Rad) at room temperature for 1 h in a 2% nonfat dry milk blocking solution in PBS-wash buffer. Finally, the membrane was washed three more times in PBS-wash buffer. Protein bands were visualized using Western Lightning Chemiluminiscence Reagent (Perkin-Elmer) and developed on Biomax Light Film (Kodak) after a 1 min exposure. When StarD5 polyclonal antibody became available, it was used for Western blot analysis at a 1:5,000 dilution under the conditions just described. A goat anti-rabbit IgG-HRP conjugate (Bio-Rad) was used as a secondary antibody at a 1:4,000 dilution. Purified StarD5 in sample buffer was used as a positive control for Western analysis with StarD5 polyclonal antibody, as freeze-thawing of the protein led to abnormal forms visualized between 40 and 50 kDa. These additional bands are not apparent with fresh protein.

Generation of [^{14}C]27-hydroxycholesterol

[^{14}C]27-hydroxycholesterol was generated using the CYP27A1 assay as described by Petrack and Latario (20) with the following modifications: 10 μl of [^{14}C]cholesterol (0.018 μl/μl) purified by the method described by Winegar et al. (21) was incubated with 300 μg of female rat liver mitochondria for 1 h at 37°C in a shaking water bath. The reaction was stopped with chloroform-methanol (2:1) (Folch method) (22). Then, [^{14}C]27-hydroxycholesterol was separated from other sterols of the chloroform phase by TLC in hexane-isopropanol-glacial acetic acid (96.5:2.5:1) solvent. The [^{14}C]27-hydroxycholesterol band was scraped and eluted with chloroform, dried under N_2, and resuspended in ethanol.

Sterol binding assays

Binding assays were performed according to the method described by Tsujishita and Hurley (7). Briefly, the purified His-tagged proteins were dialyzed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, buffer (binding buffer). Varying concentrations of the proteins were then incubated with [^{14}C]cholesterol, [^{3}H]25-hydroxycholesterol, or [^{14}C]27-hydroxycholesterol, at a final concentration of 1.76 μM, in 100 μl of binding buffer containing 2% (v/v) ethanol for 1 h at 37°C. Proteins were then immobilized on the nickel-nitrilotriacetic acid agarose resin, washed four times with binding buffer to remove unbound sterols, and eluted with the same buffer plus 1 M imidazole. The radioactivity of [^{14}C]cholesterol, [^{3}H]25-hydroxycholesterol, or [^{14}C]27-hydroxycholesterol bound to the eluted proteins was quantified with a LS60001C scintillation counter (Beckman).

Competitive binding assays were then performed using [^{3}H]25-hydroxycholesterol and [^{14}C]cholesterol at 1.76 μM. [^{14}C]cholesterol was incubated first with a solution of 1.76 μM purified protein, as already described, followed by the addition of increasing concentrations of [^{3}H]25-hydroxycholesterol. Subsequent incubation was carried out for an additional 1 h. Sterol additions were then reversed.

For unlabeled compounds (7α-hydroxycholesterol, 24-hydroxycholesterol, 24,25-hydroxycholesterol, β-sitosterol, and cholic acid), we also performed competition assays with both [^{14}C]cholesterol and [^{3}H]25-hydroxycholesterol, in which the protein was first incubated with the sterol to be tested for 1 h at 37°C and then with [^{14}C]cholesterol or [^{3}H]25-hydroxycholesterol. Finally [^{14}C]cholesterol or [^{3}H]25-hydroxycholesterol was quantified as described previously.

Circular dichroism spectroscopy

Far-ultraviolet (UV; 198–280 nm) circular dichroism (CD) measurements were carried out in a 1.0 cm path length cuvette at 400 μg protein/ml purified proteins [16.4 μM for StarD5 and 15.4 μM for (N62)StarD1] in a PBS buffer, pH 7.4, at 20°C in a Olis CD module at a scan speed of 2.7 nm/min; three spectra were averaged. Also, far-UV (184–260 nm) CD spectra were obtained with StarD5 protein at 200 μg/ml (8 μM) in the presence of cholesterol and 25-hydroxycholesterol at 2, 4, 8, and 16 μM final concentrations.

Preparation of chimeric pZEROTG-CMV/hStarD5 and CMV-StarD1 constructs and propagation

pZEROTG-CMV/hStarD5 was prepared by placing a polymerase chain reaction fragment containing the coding region for human StarD5 into the pZEROTG-CMV expression vector opened

with *Eco*RV and dephosphorylated with Calf Intestinal Phosphatase. The primers used for the reaction were 5′-GCTCCCGACGCAATG-GACCC-3′ and 5′-CACGGGAGTTCTTTGCCAAG-3′. Five micrograms of human liver total RNA in a final volume of 20 μl was used as template in an RT reaction using 100 pmol of random hexamers as primers and 1.5 mM Mg for 10 min at 25°C and 1 h at 42°C. Five microliters of the RT reaction was used as template in the PCR. The PCR conditions were as follows: 5 min at 98°C, 5 min at 72°C (1 cycle), followed by 35 cycles of 1 min at 94°C, 1 min at 62°C, and 1.5 min at 72°C. The reaction was finished with one cycle of 10 min at 72°C. The pZEROTG-CMV expression vector was obtained from Dr. Kris Valerie's laboratory at Virginia Commonwealth University. The CMV-StarD1 adenovirus was constructed as described previously (23).

Recombinant virus CMV-StarD1 and CMV-StarD5 were transfected into human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA). Adenovirus DNA from the resulting plaques was further screened by Southern blotting for the presence of the inserts. To purify the recombinant virus, the crude supernatant was carefully layered over a two-step CsCl gradient as described previously (23).

Isolation of primary rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (250–300 g) as described previously (24) using the collagenase perfusion technique of Bissell and Guzelian (25).

Infection with adenovirus and filipin staining of cholesterol in hepatocytes

Primary rat hepatocytes were isolated and plated as described previously at 15–20% of normal density in Williams media with insulin and dexamethasome on six-well culture plates containing cover slips and incubated at 37°C and 5% CO_2. Twenty-four hours after plating, the cells were infected with either unpurified recombinant adenovirus encoding StarD1, StarD5, or Ad-CMV control virus. After 2 h of infection, media were removed and replaced with fresh media. The next day, media were removed and the cells were washed twice with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at 4°C. The cells were then washed three times, 5 min each, with PBS while rocking gently at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at 4°C, stained with 5 mg/ml filipin in PBS plus 0.5% BSA for 30 min at 37°C, and washed three times, 5 min each, with PBS while rocking gently at room temperature in the dark. The cover slips containing the cells were taken from the wells and mounted onto glass slides. The cells were allowed to dry for at least 45 min before being placed on a "fluorescence" microscope with excitation filter 360/40 nm, emission filter 460/50 nm, and beam splitter 400 nm.

Integrated optical density determination

Fluorescence was quantified via Image-Pro® Plus analysis software and expressed as integrated optical density.

Preparation of fractions from human liver

For mitochondrial isolation, liver tissue was homogenized in 9 volumes (10% homogenate) of a solution of 0.25 M sucrose, 0.5 mM EDTA, and 0.1 M KH_2PO_4, pH 7.4. The homogenate was centrifuged at 600 *g* for 15 min. The supernatant was centrifuged for an additional 20 min at 6,500 *g*. The mitochondria-enriched pellet was resuspended in 0.5 volume of initial buffer and centrifuged at 6,500 *g* for 20 min. This step was repeated in one-fourth the initial volume before being centrifuged a third time for 20 min at 6,500 *g*. The mitochondrial pellet was then suspended in a one-tenth volume of 0.1 M Tris-HCl, pH 7.7, at room temperature.

For microsomal and cytosolic fraction isolation, 1 g of human liver was homogenized in 5 ml of ice-cold 200 mM potassium buffer (pH 7.2) containing 100 mM sucrose, 50 mM KCl, 1 mM EDTA, and 50 mM NaF (isolation buffer). The homogenate was centrifuged at 10,000 rpm in a centrifuge at 4°C for 15 min. The pellet (nucleus and cell debris) was resuspended into 5 ml of isolation buffer, and the supernatant was decanted and centrifuged at 105,000 *g* for 2 h at 4°C. The microsomal pellet was resuspended into 1 ml of isolation buffer. Fractions were stored at −80°C until use. Protein determination was carried out with the Bio-Rad Miniprotein Assay.

Preparation of fractions from primary rat hepatocytes

Primary rat hepatocytes (25×10^6) were harvested at 24 h after infection with either unpurified recombinant adenovirus encoding StarD5 or Ad-CMV control virus in 1 ml of ice-cold isolation buffer. Cells were ruptured by sonication for 10 s, and the cell lysate was centrifuged at 10,000 rpm in a microcentrifuge at 4°C for 15 min. The pellet (nucleus and cell debris) was resuspended into 1 ml of isolation buffer, and the supernatant was decanted and centrifuged at 105,000 *g* for 2 h at 4°C. The microsomal pellet was resuspended into 1 ml of isolation buffer. Fractions were

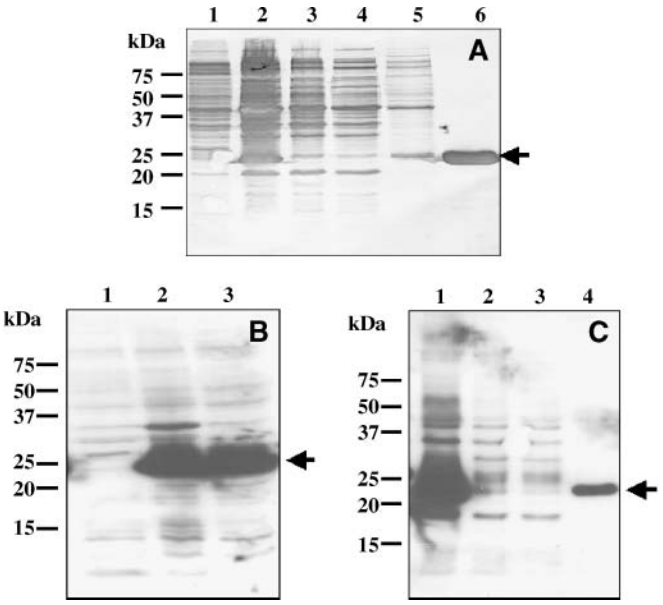

Fig. 1. Purification and identification of recombinant human StarD5 protein. A: SDS-PAGE analysis and Coomassie blue staining of human StarD5 overexpressed in BL21(DE3) cells at each step of the purification. Lane 1, total soluble protein before induction (30 μg); lane 2, total soluble protein after induction (50 μg); lane 3, total soluble protein after incubation with the resin (30 μg); lane 4, proteins eluted with wash buffer I (30 μg); lane 5, proteins eluted with wash buffer II (10 μg); lane 6, protein eluted with lysis buffer plus 1 M imidazole (20 μg). B: Western blot analysis of human StarD5 expressed in BL21(DE3) cells with monoclonal anti-polyhistidine antibody. Lane 1, total soluble protein before induction (60 μg); lane 2, inclusion body protein after induction (60 μg); lane 3, total soluble protein after induction (60 μg). C: Western blot analysis of human StarD5 purification with monoclonal anti-polyhistidine antibody. Lane 1, total soluble protein after induction (50 μg); lane 2, total soluble protein after incubation with the resin (20 μg); lane 3, proteins eluted with wash buffer II (10 μg); lane 4, protein eluted with lysis buffer plus 1 M imidazole (200 ng). In all panels, the arrow indicates a major protein band found at ~24.5 kDa.

stored at −80°C until use. Protein determinations for each fraction were carried out with the Bio-Rad Miniprotein Assay, and no statistical differences were noted in like fractions.

Extraction and HPLC of cholesterol

Sterols were extracted from the different cellular fractions with chloroform-methanol (2:1, v/v) as described by Folch et al. (22). After the addition of water and phase separation, the chloroform phase containing the cholesterol was removed and evaporated under a stream of N_2 at room temperature. For the quantitative determination of total cholesterol, the residue obtained was assayed with the Wako Cholesterol CII assay (Wako Chemicals USA, Richmond, VA), an in vitro, enzymatic, colorimetric assay. For the determination of free cholesterol, the residue obtained was dissolved in 500 μl of a 100 mM phosphate buffer, pH 7.5, gently vortexed, and treated with 2 units of cholesterol oxidase for 30 min at 37°C. The reaction was terminated by the addition of 1.5 ml of methanol. Cholesterol was extracted with 2 × 3 ml of hexane, and the extracts were evaporated under N_2 at room temperature. The residue was dissolved in 100 μl of hexane and analyzed by HPLC in a normal phase silica column with hexane-isopropanol-acetic acid (965:25:10, v/v) at 22°C and a flow rate of 1 ml/min. Free cholesterol was identified by comparison with known cholesterol standards and quantified using peak area ratios.

Statistics

Data from sterol binding, filipin staining, and free cholesterol concentrations in primary rat hepatocytes are reported as means ± SD of three separate experiments.

RESULTS

Purification and characterization of the recombinant human (N62)StarD1 and StarD5 proteins, and preparation of antibody against StarD5

StarD5 protein expression resulted in a 24.5 kDa major protein band by SDS-PAGE analysis (**Fig. 1A**). Approximately 50% of the protein was in the soluble fraction and had a His-tag, as shown by Western blot with monoclonal antibody against polyhistidine (Fig. 1B). From 585 mg of total soluble cell protein, 1 liter culture, 16.3 mg of His-tag/protein was purified. Recombinant His-tag/protein accounted for 7% of the total soluble cell protein, which corresponds to 41 mg of His-tag/StarD5. The yield was 40%, calculated as milligrams of pure His-tag/protein obtained, divided by the total His-tag/protein in the soluble cell fraction. The pure protein obtained was immunoreactive to a monoclonal antibody against polyhistidine, as determined by Western blot analysis (Fig. 1C). Finally, the purified protein was analyzed by N-terminal sequencing, and after seven cycles of Edman degradation, the sequence obtained was Met-Asp-Pro-Ala-Leu-Ala-Ala, which is identical to the N-terminal sequence of the human StarD5 protein. After purification, 2.5 mg of His-tag/StarD5 was used to generate polyclonal antibodies in rabbits (Sigma-Genosys) and used for Western blot analysis. For the recombi-

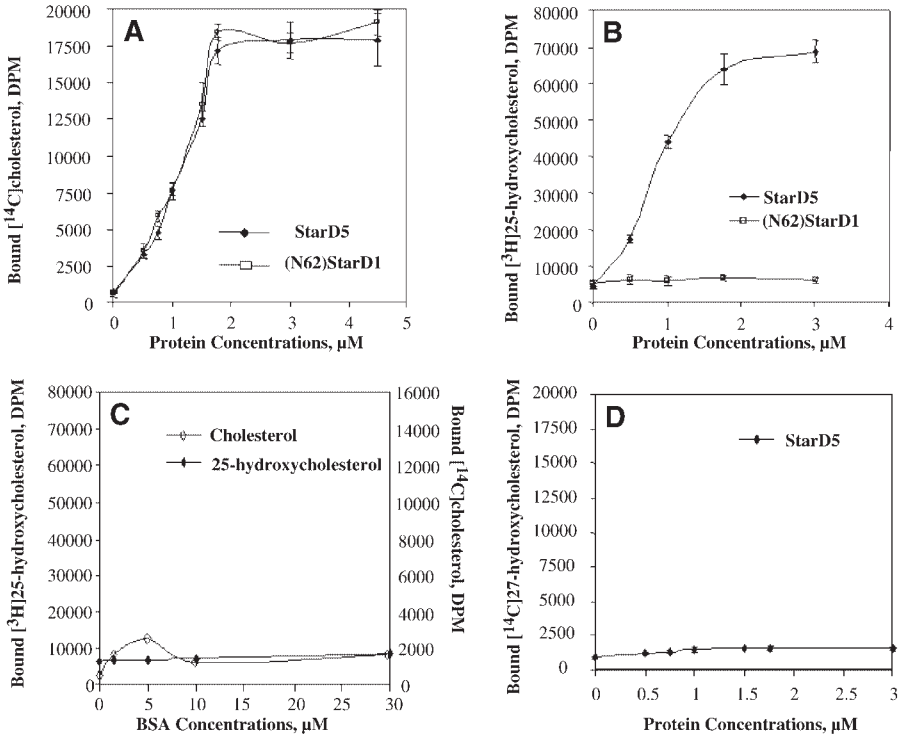

Fig. 2. Characterization of StarD5 binding capabilities with cholesterol and oxysterols. Different concentrations of His-tagged (N62)StarD1 or StarD5 were incubated with 1.76 μM [^{14}C]cholesterol (A) or [^{3}H]25-hydroxycholesterol (B) for 1 h at 37°C. C: A control procedure was performed with different concentrations of BSA incubated with 1.76 mM [^{14}C]cholesterol (open diamonds) or [^{3}H]25-hydroxycholesterol (closed diamonds) for 1 h at 37°C. D: [^{14}C]27-hydroxycholesterol binding assay for StarD5 was performed as already described. Radioactivity of [^{14}C]cholesterol, [^{3}H]25-hydroxycholesterol, or [^{14}C]27-hydroxycholesterol bound to eluted proteins was quantified. Data are presented as means ± SD of three independent experiments.

nant His-tag/(N62)StarD1 protein, similar results were obtained, and the N-terminal sequence of the protein was confirmed by Edman degradation.

(N62)StarD1 and StarD5 containing START domains bind cholesterol

Tsujishita and Hurley (7) reported data showing direct binding of cholesterol by a His-tagged StarD1 (full-length protein). To determine whether (N62)StarD1 and StarD5 His-tagged proteins were capable of binding cholesterol, the same method was used. At a 1.76 μM protein concentration with both proteins, all [^{14}C]cholesterol added was bound to the proteins, at a stoichiometry of 1:1 for (N62) StarD1 and 1:1 for StarD5 (**Fig. 2A**). As a negative control, assays were performed with (N62)StarD1 and StarD5 that had been heat-inactivated. Neither heat-inactivated protein was capable of binding cholesterol (data not shown). Assays performed with StarD5 and [^{3}H]25-hydroxycholesterol showed that StarD5 was capable of binding the oxysterol at a stoichiometry of 1:1 (Fig. 2B). The same assay was carried out with bovine albumin at different concentrations, with no evidence of nonspecific binding to the resin observed with [^{14}C]cholesterol or [^{3}H]25-hydroxycholesterol (Fig. 2C). A direct binding assay using StarD5 with [^{14}C]27-hydroxycholesterol (Fig. 2D) or competition assays using StarD5 with 7α-hydroxycholesterol, 24-hydroxycholesterol, 24,25-hydroxycholesterol, β-sitosterol, or cholic acid (data not shown) did not show any evidence of binding. Competitive binding assays using [^{3}H]25-hydroxycholesterol and [^{14}C]cholesterol demonstrated a concentration-dependent displacement of [^{3}H]25-hydroxycholesterol by [^{14}C]cholesterol. [^{3}H]25-hydroxycholesterol, however, was unable to displace [^{14}C]cholesterol (data not shown). For comparative purposes, the binding affinity of (N62)StarD1 was tested for [^{3}H]25-hydroxycholesterol. No detectable binding was found (Fig. 2B).

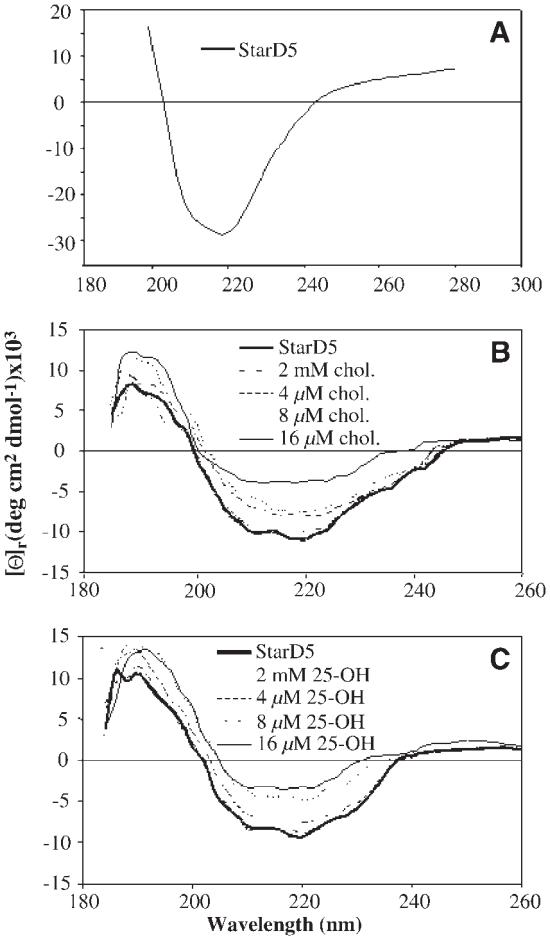

Fig. 3. Far-ultraviolet CD spectrum of StarD5. The data shown are averages of three scans of the recombinant protein human StarD5 at pH 7.4, 20°C, alone at a concentration of 400 μg/ml (A), or 200 μg/ml StarD5 with different amounts of cholesterol (B) or 25-hydroxycholesterol (C).

(N62)StarD1 and StarD5 CD spectroscopy

The CD spectrum of the (N62)StarD1 protein at pH 7.4 gave minima of ~208 and 222 nm (data not shown), as described previously by Bose et al. (26, 27), suggestive of the presence of many α-helical structures in the protein. The CD spectrum of the StarD5 protein gave a minimum at 218 nm (**Fig. 3A**), suggestive of the presence of a large number of β-sheet structures rather than other secondary structures (i.e., random coils and α-helices) (28). Far-UV CD spectra of StarD5 incubated with different concentrations of cholesterol (Fig. 3B) showed dose-dependent changes in the ellipticity at 218 nm, consistent with StarD5 binding of cholesterol as shown in the sterol binding assays. Although requiring a greater concentration than for cholesterol, the far-UV CD spectra obtained with StarD5 in the presence of 25-hydroxycholesterol (Fig. 3C) showed changes in the ellipticity at 218 nm, again consistent with StarD5's binding of 25-hydroxycholesterol as shown in the sterol binding assays.

StarD5 detection

StarD5 was detected in human liver (**Fig. 4A**) using the StarD5 polyclonal antibody. The expression level of StarD5 in human liver tissue was 714 ng/mg liver protein, as calculated from data in Fig. 4A. Human liver fractions were then examined by Western blot analysis to localize StarD5. StarD5 protein was detected in the cytosolic fraction but not in mitochondrial or microsomal fractions (Fig. 4B).

Overexpression of StarD5 increases intracellular free cholesterol in hepatocytes

Filipin staining in primary rat hepatocytes overexpressing human StarD5 protein showed a significantly greater increase in intracellular free cholesterol than in hepatocytes infected with adenovirus encoding StarD1 or Ad-CMV control virus (**Fig. 5A**). Quantitative analysis of fluorescence within cells infected with the StarD5 virus showed a 12-fold increase over controls ($P < 0.01$; Fig. 5B). Only a trend toward increased fluorescence was observed after StarD1 overexpression (~1.5-fold; NS; Fig. 5B).

Levels of free cholesterol in the different cellular fractions of primary rat hepatocytes infected with recombi-

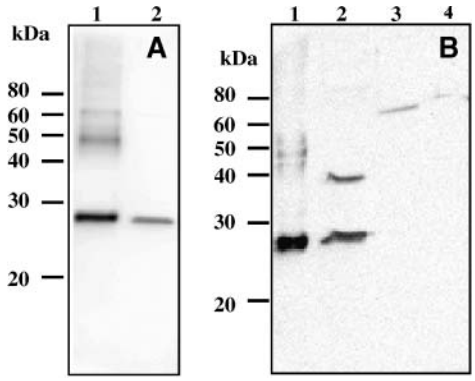

Fig. 4. Distribution of StarD5 protein in human liver. A: Western blot analysis with StarD5 polyclonal antibody. Lane 1, StarD5, pure protein (150 ng); lane 2, human liver, total protein (70 μg). StarD5 protein was detected in all of the samples analyzed. B: Western blot analysis with StarD5 polyclonal antibody was carried out with different fractions isolated from human liver. Lane 1, StarD5, pure protein (200 ng); lane 2, cytosolic fraction (25 μg); lane 3, mitochondrial fraction (25 μg); lane 4, microsomal fraction (25 μg). StarD5 protein was detected only in the cytosolic fraction of the liver.

nant adenovirus encoding StarD5 or control virus were then quantified by HPLC (**Fig. 6**). Compared with controls, StarD5 overexpression in primary rat hepatocytes was associated with significantly increased levels of free cholesterol (3.5-fold; $P < 0.01$) in the microsomal fraction, whereas free cholesterol levels in the "pellet" and cytosolic fractions of StarD5-overexpressing cells were decreased (Fig. 6). Levels of total cholesterol were also determined for comparative purposes, and no significant differences were seen in control versus after StarD5 overexpression (162 ± 8 vs. 169 ± 11 μg/mg protein, respectively).

DISCUSSION

To pursue functional studies of the role of StarD5 on lipid transport, the human StarD5 protein was purified and used to generate a polyclonal antibody against StarD5. Using this antibody, StarD5 protein was detected in human liver (Fig. 4), confirming earlier mRNA expression in mouse liver (2, 6).

Interestingly, the StarD5 protein, like other members of the StarD4 subfamily, does not exhibit an N-terminal domain that would direct the protein to specific cellular organelles. Therefore, StarD5 was predicted to be a cytoplasmic protein (6). To confirm this, human liver cell fractions were examined by Western blot analysis using the polyclonal antibody against StarD5. The StarD5 protein was localized to the cytosolic fraction (Fig. 4B) with no association with mitochondria, endoplasmic reticulum membranes, or other organelles. This finding suggested that StarD5 is a soluble protein in the cytosol, differing from the START domain proteins, StarD1 and StarD3, which have a mem-

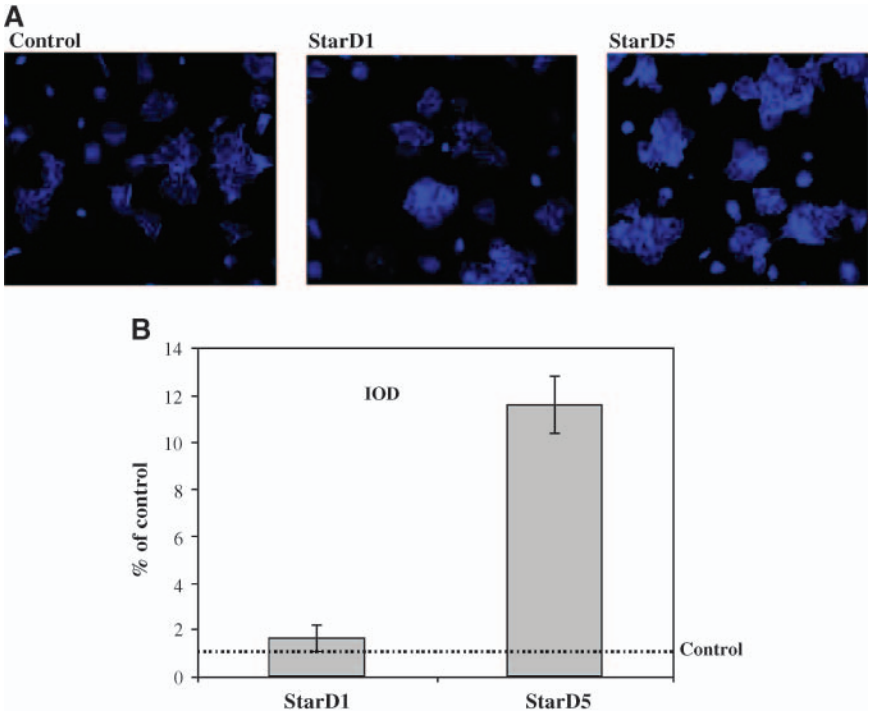

Fig. 5. Filipin staining for localization of free cholesterol in primary rat hepatocytes. A: Control, cells infected with Ad-CMV control virus; StarD1, cells infected with adenovirus encoding human StarD1; StarD5, cells infected with adenovirus encoding human StarD5. B: Integrated optical density (IOD). Fluorescence (brightness) in overexpressed StarD1 and StarD5 samples compared with control. Data are presented as means ± SD of three independent experiments.

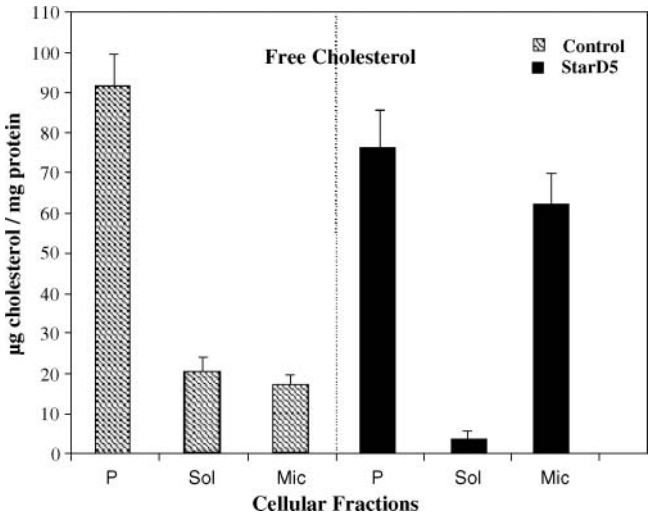

Fig. 6. Level of free cholesterol in different cellular fractions from primary rat hepatocytes infected with recombinant adenovirus encoding StarD5 or Ad-CMV control virus. Levels of free cholesterol in the pellet (P, nucleus and cell debris), soluble fraction (Sol), and microsomes (Mic) of primary rat hepatocytes after infection with the mentioned recombinant adenovirus. Data are presented as means ± SD of three independent experiments.

brane binding domain and are membrane binding proteins.

Although StarD5 does not have an N-terminal targeting sequence, it has a START domain and is predicted to be able to bind cholesterol and/or other sterols. The assays reported here show that StarD5 binds cholesterol with a similar stoichiometry to that reported for StarD1 (7) and (N62)StarD1 (Fig. 3A). In direct contrast to StarD1, which was not shown to bind 25-hydroxycholesterol, similar assays performed with StarD5 showed binding of this molecule with a similar stoichiometry to that of cholesterol (Fig. 3B). These results implicate StarD5 not only as a cholesterol transporter, but also as capable of binding and transporting other sterol-derived molecules related to the cholesterol/bile acid biosynthetic pathways. Although it has been reported that different proteins with a START domain are capable of selectively binding different lipids (7, 13) and may even have additional roles (3, 5), the ability of StarD5 protein to bind different molecules represents a finding not reported previously for a protein with a START domain. The saturable binding of cholesterol and 25-hydroxycholesterol at stoichiometry 1:1 with StarD5 suggests that binding occurs at a specific site and not as a result of nonspecific aggregation of protein with sterols. The lack of competitive displacement of cholesterol by 25-hydroxycholesterol is indicative of a higher affinity of StarD5 for cholesterol than 25-hydroxycholesterol. Interestingly, additional StarD5 binding studies failed to show StarD5 binding of any other tested metabolic or plant sterols.

CD spectroscopy of the (N62)StarD1 protein gave two minima (208 and 222 nm), as shown previously by Bose, Baldwin, and Miller (26), confirming that the protein adopted a normal structure. The StarD5 protein gave a minimum at 218 nm, suggesting a primarily β-sheet character for this protein. Although the StarD5 structure is unknown, the structure of mouse StarD4 protein, a protein that also belongs to the StarD4 subfamily (6), also has a large number of β-sheets (12). These data suggested that, like (N62)StarD1, the StarD5 protein adopted a normal conformation after purification. Incubation of StarD5 with cholesterol elicited changes in the spectrum of the protein. The minimum at 218 was lost even at the lowest concentration of added cholesterol, indicating major StarD5 conformational changes with cholesterol binding to the protein. Although still consistent with StarD5's binding of 25-hydroxycholesterol, the far-UV spectra of StarD5 incubated with 25-hydroxycholesterol (Fig. 4B) showed that the 218 nm minimum was not lost completely until higher concentrations of 25-hydroxycholesterol were added.

Filipin staining (a well-described marker for the staining of intracellular membrane-associated free cholesterol) (29) of hepatocytes overexpressing StarD5 gave us a novel approach for determining under more physiologic conditions the ability of StarD5 to affect intracellular levels of free cholesterol. These results provide physiologic evidence for the ability of StarD5 to bind and transfer free cholesterol intracellularly, although the mechanism remains unknown.

Just as importantly, the results obtained from the quantification of free cholesterol by HPLC showed a marked increase in the levels of free cholesterol in the microsomal fraction after StarD5 overexpression. This corroborates the increase in fluorescence by filipin staining of the intracellular free cholesterol. These results, in conjunction with the sterol binding assays and CD spectra of cholesterol-induced conformational changes, are further evidence for a StAR-like cholesterol transfer activity of StarD5, which appears to be directed to the microsomes.

Although the physiologic role of StarD5 remains uncertain, the selectivity of StarD5 for two sterols, compared with one for StarD1, raises basic questions about the function of this sterol binding protein. The oxysterol, 25-hydroxycholesterol, although endogenously generated, is believed to be mostly derived in humans through autoxidation; it has been shown to be not only a potent regulatory oxysterol under in vitro conditions but an important inflammatory mediator of atherogenesis (30, 31). Studies including protein crystallization, the generation of StarD5 knockout conditions, and further cellular immunohistochemistry/cytochemistry are currently under way to elucidate the physiological role and regulation of this novel cholesterol and oxysterol binding protein.

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