Steroidogenic Acute Regulatory Protein (StAR) Is A Sterol Transfer Protein*

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Steroidogenic acute regulatory protein (StAR) plays a critical role in steroidogenesis by enhancing the delivery of substrate cholesterol from the outer mitochondrial membrane to the cholesterol side chain cleavage enzyme system on the inner membrane. A recombinant StAR protein lacking the first N-terminal 62 amino acid residues that includes the mitochondrial targeting sequence was shown to stimulate the transfer of cholesterol and β-sitosterol from liposomes to heat-treated mitochondria in a dose-, time-, and temperature-dependent manner. A recombinant mutant StAR protein that cannot stimulate steroidogenesis by isolated mitochondria did not promote sterol transfer. Unlike the more promiscuous lipid transfer protein, sterol carrier protein 2 (SCP2), StAR did not stimulate phosphatidylcholine transfer in our assay system. The recombinant StAR protein increased cholesterol transfer to heat-treated microsomes as well as to heat- and trypsin-treated mitochondria. These observations demonstrate that StAR has sterol transfer activity, which may reflect its ability to enhance desorption of cholesterol from sterol-rich donor membranes. We suggest that the ability of StAR to promote sterol transfer explains its steroidogenic activity.

Steroidogenic acute regulatory protein (StAR) plays a significant role in steroidogenesis at the initial step in hormone biosynthesis, the conversion of cholesterol into pregnenolone (1–5). This reaction is catalyzed by the cholesterol side chain cleavage enzyme, which is localized to the mitochondrial inner membranes. StAR appears to increase the delivery of sterol substrate from the outer mitochondrial membrane to the cholesterol side chain cleavage enzyme (P450scc) because a StAR mutant lacking the first 62 N-terminal amino acid residues (N-62 StAR) is as steroidogenically active as the full-length protein when expressed in COS-1 cells in conjunction with the human cholesterol side chain cleavage system (10, 11). Moreover, recombinant N-62 StAR protein, despite the fact that it is incapable of being imported into mitochondria, stimulates pregnenolone synthesis by mitochondria isolated from bovine corpus luteum (11). These observations have been interpreted as indicating that StAR acts on the outer mitochondrial membrane to promote cholesterol delivery to cytochrome P450scc. The mechanism by which StAR acts to enhance substrate delivery to the cholesterol side chain cleavage enzyme has eluded investigators, and no mitochondrial receptor has yet been identified for the StAR protein. The present experiments were carried out to test the hypothesis that StAR is a sterol transfer protein. To this end, we examined the ability of recombinant N-62 StAR to stimulate the movement of lipids from donor vesicles to acceptor membranes. Our findings demonstrate that StAR promotes sterol but not phosphatidylcholine transfer, distinguishing its activity from other known lipid transfer proteins.

MATERIALS AND METHODS

Recombinant StAR and SCP2 Proteins—N-62 StAR containing a C-terminal or N-terminal 6-histidine tag (His6tag), and a N-62 StAR C-terminal His6 tag mutant with an A218V amino acid replacement were produced in Escherichia coli and purified as described previously (11). The A218V mutation was selected because it inactivates the protein and causes congenital adrenal lipoid hyperplasia (7). Our previous studies demonstrated that N-62 C-His6 tag StAR stimulates pregnenolone synthesis by isolated bovine corpus luteum mitochondria, whereas the N-62 C-His6 tag A218V mutant is inactive (11).

Recombinant human mature sterol carrier protein 2 (SCP2) was produced in E. coli and purified as described by Matsuura et al. (12). The N-terminal amino acid residue of the recombinant SCP2 was an alanine rather than serine, a change engineered in the SCP2 cDNA sequence to enhance translation. The recombinant SCP2 employed has previously been shown to have lipid transfer activity (12).

Lipid Transfer Assays—The lipid transfer activities of recombinant StAR and SCP2 were examined using methods previously described (12–14) that entail the measurement of transfer of 3H-labeled lipids (1.0 μCi/μmol) incorporated into egg yolk phosphatidylcholine liposomes to heat-treated mitochondria or microsomes. The liposomes contained tracer amounts of 3H[1]H]cholesterol to monitor lipid-acceptor fusion or liposome trapping. Briefly, liposomes were prepared by sonication as described by Bloj and Zilversmit (13). Sterol-containing liposomes had a molar ratio of phosphatidylcholine/sterol of 1:0.875. In each assay, donor liposomes contained 16 nmol of phosphatidylcholine and 14 nmol of cholesterol or β-sitosterol (sterol transfer) or 30 nmol of phosphatidylcholine (phospholipid transfer). The liposomes produced by this method have a mean diameter between 30 and 45 nm (14).

Acceptor organelles were prepared by differential centrifugation (14). Mitochondria were isolated from mouse and rat liver, bovine corpus...
and yeast. Microsomes were prepared from the post-mitochondrial supernatant of rat liver homogenates. 3 mg of the organelle protein was added as acceptor in each assay in a final volume of 500 \( \mu l \) 20 mM Tris buffer containing 250 mM sucrose, 1 mM EDTA, and 1 mg of bovine serum albumin, pH 7.4, with the indicated concentration of recombinant N-62 His6 tag StAR or SCP2. The organelle preparations were pre-heated at 60 °C for 30 min to denature endogenous lipases as described previously (13). In selected experiments, the heat-treated mitochondria were also treated with trypsin (200 \( \mu g/ml \)) for 40 min at 4 °C. The trypsin was then neutralized with 1 mM phenylmethylsulfonyl fluoride and 1.25 mg/ml soybean trypsin inhibitor for 10 min at 4 °C prior to the transfer assay. The data presented in Figs. 1, 2, and 4 represent the stimulation of lipid transfer over that occurring in the absence of added recombinant protein. In addition, all results presented in these figures were corrected for the small amount of liposome-acceptor fusion/trapping detected by the transfer of \( ^{3}H \) triolein, which always amounted to \( <10\% \), and usually \( \leq3\% \), of N-62 StAR- or SCP2-stimulated lipid transfer. Experiments were reproduced on at least three separate occasions except where noted. Values presented are the means ± S.E.

**RESULTS**

**C-His6 tag N-62 StAR stimulates cholesterol transfer.** Egg phosphatidylcholine liposomes containing \( ^{14}C \) cholesterol were incubated with the indicated concentration of C-His6 tag N-62 StAR and heat-treated mitochondria for 20 min at 37 °C, and the percentage of transfer of \( ^{14}C \) cholesterol was determined as described in the text. Values are the means ± S.E. from three separate experiments representing the percentage of transfer over that observed in the absence of recombinant protein, corrected for apparent transfer due to trapping or fusion of liposomes with acceptor membranes.

**Fig. 1.** A, dose dependence of C-His6 tag N-62 StAR-promoted sterol transfer. Egg phosphatidylcholine liposomes containing \( ^{14}C \) cholesterol were incubated with the indicated concentration of C-His6 tag N-62 StAR and heat-treated mitochondria for 20 min at 37 °C, and the percentage of transfer of \( ^{14}C \) cholesterol was determined as described in the text. Values are the means ± S.E. from three separate experiments representing the percentage of transfer over that observed in the absence of recombinant protein, corrected for apparent transfer due to trapping or fusion of liposomes with acceptor membranes. B, time course of \( ^{14}C \) cholesterol transfer. \( ^{14}C \) Cholesterol-egg phosphatidylcholine liposomes were incubated with 1 \( \mu M \) C-His6 tag N-62 StAR and heat-treated mitochondria for the indicated times at 37 °C.

**Western Blotting—Western blot analysis of mitochondrial and microsomal protein markers was carried out as described by Towbin et al. (15). Endosomal transferrin receptors were detected with a mouse anti-transferrin receptor antibody purchased from Zymed Laboratories Inc. (South San Francisco, CA), whereas mitochondrial heat shock protein-70 (HSP70) was detected with a mouse anti-HSP70 antibody obtained from Affinity BioReagents, Inc. (Golden, CO). Antibody detection was performed using horseradish peroxidase-conjugated anti-mouse antibodies purchased from Cappel (Durham, NC) and an enhanced chemiluminescence kit from Amersham Pharmacia Biotech.
was reached at 1–4 μM STAR protein, resulting in 60% cholesterol transfer from donor vesicles to the heat-treated mitochondria. The incubation medium in all cases contained 2 mg/ml bovine serum albumin, and the reported lipid transfer activities in Fig. 1 reflect sterol transfer above that occurring in the absence of recombinant protein. C-His6 tag N-62 StAR stimulated sterol transfer to heat-treated mouse and rat liver, bovine corpus luteum, and yeast mitochondria to a similar extent (data not shown). Hence, rat liver mitochondria were used in most of the studies.

The action of C-His6 tag N-62 StAR on cholesterol transfer was rapid, with the majority of transfer occurring within 5 min of incubation at 37 °C. The cholesterol transfer activity of the C-His6 tag recombinant N-62 STAR protein was temperature-dependent because cholesterol transfer was minimal at 4 °C (Fig. 2). N-62 C-His6 tag N-62 STAR also displayed cholesterol transfer activity (Fig. 2), as did recombinant human SCP2 at a 1 μM concentration. The former finding demonstrates that the position of the His6 tag does not substantively influence sterol transfer activity of N-62 STAR. In contrast, heat-denatured C-His6 tag N-62 STAR and the C-His6 tag N-62 A218V mutant displayed minimal cholesterol transfer activity (<2.5 and <1% sterol transfer, respectively), even when added at concentrations greater than the wild-type recombinant protein (Fig. 2).

Importantly, C-His6 tag N-62 STAR increased cholesterol transfer to heat-treated mitochondria that had been pre-treated with trypsin to digest outer mitochondrial membrane proteins. The efficacy of the trypsin treatment was documented by SDS-polyacrylamide gel electrophoresis analysis, which revealed substantial degradation of mitochondrial proteins (Fig. 3).

C-His6 tag N-62 StAR increased the transfer of cholesterol to heat-treated microsomes, as did SCP2 (Fig. 4). In contrast to SCP2, also called nonspecific lipid transfer protein because it promotes the transfer of a variety of lipids including various sterols, phospholipids, and glycolipids, N-62 C-His6 tag STAR did not stimulate phosphatidylcholine transfer to mitochondria or to microsomes (Fig. 4). The microsomal preparation was shown to have modest mitochondrial contamination by Western blot analysis of mitochondrial heat shock protein-70 (Fig. 5). The mitochondrial preparation did not contain detectable levels of the transferrin receptor, a microsomal/endosomal marker (Fig. 5).

The specificity of C-His6 tag N-62 StAR sterol transfer activity was tested by examining the ability of the protein to promote transfer of the plant sterol, β-sitosterol. The C-His N-62 STAR protein enhanced β-sitosterol transfer from sterol-rich liposomes to heat-treated mitochondria to the same extent as it promotes cholesterol transfer (Table I).
Our observations demonstrate that N-62 StAR has sterol transfer activity with specificity that can be distinguished from SCP₂, a protein that stimulates the transfer of several different lipids from donor vesicles to acceptor membranes. The fact that recombinant N-62 C-His₆ tag StAR effectively stimulates sterol transfer from sterol-rich liposomes to heat-treated mitochondria isolated from various animal sources, to mitochondria that were trypsin-treated, and to microsomes demonstrates that StAR protein lacking the N-terminal mitochondrial targeting sequence does not have acceptor organelle specificity and that it does not require heat- and/or trypsin-sensitive proteins on the acceptor for efficient sterol transfer. Indeed, these observations suggest that the specificity of action of the StAR protein is determined by the N-terminal mitochondrial targeting sequence. These conclusions support a bipartite model of functional domains in StAR: an organelle-specifying N terminus and a sterol transferring activity encoded by the remaining C terminus of the protein. StAR, like SCP₂, does not distinguish between cholesterol and the plant sterol, β-sitosterol, a cholesterol structure with an ethyl group at carbon 24. However, further experiments are needed to determine whether the sterol transfer activity of StAR differentiates other features of the cholesterol molecule, including the 3-oxo function and the Δ⁵ double bond.

StAR is a phosphoprotein (4, 5), and the phosphorylation state of StAR is directly correlated with steroidogenesis (16, 17). We have previously shown that the ability of StAR to be phosphorylated at serine residue 195, which is in the context of a consensus protein kinase A phosphorylation motif that is conserved in all species of StAR identified to date, is associated with increased steroidogenic activity (18). Because a StAR mutant that cannot be phosphorylated on serine 195 (S195A) had approximately 40% of the steroidogenic activity of the wild-type protein in transfected COS-1 cells, phosphorylation of StAR is evidently not obligatory for activity of StAR. The recombinant StAR protein used in the present studies is not phosphorylated. Thus, we can conclude that StAR does not need to be posttranslationally modified to promote sterol transfer. It should also be noted that at present we do not know what percentage of the recombinant C-His₆ tag N-62 StAR is properly folded and biologically active. Thus, the native protein may be active in promoting sterol transfer at much lower concentrations than those employed in the experiments described here.

Because StAR is not found in large quantities in steroidogenic cells, a 1:1 carrier mechanism for sterol delivery to the inner mitochondrial membrane is unattactive. The promotion of membrane fusion by StAR is excluded as a mechanism because the amount of lipid transfer in our assays attributable to a fusion process was minimal. This is not consistent with models of StAR action based on a fusogenic role for the protein. A mechanism involving formation of bridges between donor and acceptor membranes, as has been suggested previously for SCP₂ action (14), is also unlikely because StAR evidently can act on the outer mitochondrial membrane and thus could not easily be envisioned to form bridges between the outer and inner mitochondrial membranes. Therefore, we favor a "cata-lytic" process by which StAR promotes multiple molecules of sterol to move from the outer to the inner mitochondrial membranes. StAR perturbation of donor membrane structure, resulting in sterol desorption from the relatively cholesterol-rich outer mitochondrial membrane (19) is a possible mechanism for substrate transfer to the cholesterol-poor inner mitochondrial membrane. This mechanism predicts that the direction of sterol flux is influenced by the abundance of sterol in the two membranes with net sterol transfer slowing or ceasing as the sterol contents of the donor and acceptor membranes equilibrate. In mitochondria, cholesterol provoked to exit the outer membrane by StAR might travel to inner the membrane via pre-existing contact sites.

Enhanced sterol desorption has also been proposed as the explanation for the ability of SCP₂ to increase sterol transfer in cell-free assays (20). However, because StAR displays greater specificity for sterol transfer than SCP₂, it is likely that the two proteins act by different molecular mechanisms. It has been attractive to envision StAR interacting with a protein "receptor" or recognition protein on the outer mitochondrial membrane. However, the fact that StAR can promote steroidogenesis by mitochondria of cells that do not normally produce steroid hormones (i.e., COS-1 cells transfected with the cholesterol side chain cleavage enzyme system) and that StAR can stimulate cholesterol transfer to mitochondrial as well as microsomal membranes argues against the existence of a receptor on mitochondria of cellular sterols for StAR-mediated steroidogenesis. In summary, the present studies demonstrate that StAR has sterol transfer activity that may account for its steroidogenic properties.

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