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Effect of Truncated Forms of the Steroidogenic Acute Regulatory Protein on Intramitochondrial Cholesterol Transfer*

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ABSTRACT

It has been proposed that the steroidogenic acute regulatory (StAR) protein controls hormone-stimulated steroid production by mediating cholesterol transfer to the mitochondrial inner membrane. This study was conducted to determine the effect of wild-type StAR and several modified forms of StAR on intramitochondrial cholesterol transfer. Forty-seven N-terminal or 28 C-terminal amino acids of the StAR protein were removed, and COS-1 cells were transfected with pCMV vector only, wild-type StAR, N-47, or the C-28 constructs. Lysates from the transfected COS-1 cells were then incubated with mitochondria from MA-10 mouse Leydig tumor cells that were preloaded with [^{3}H]cholesterol. After incubation, mitochondria were collected and fractionated on sucrose gradients into outer membranes, inner membranes, and membrane contact sites, and [^{3}H]cholesterol content was determined in each membrane fraction. Incubation of MA-10 mitochondria with wild-type StAR containing cell lysate resulted in a significant 34.9% increase in [^{3}H]cholesterol content in contact sites and a significant 32.8% increase in inner mitochondrial membranes. Incubations with cell lysate containing N-47 StAR protein also resulted in a 16.4% increase in [^{3}H]cholesterol in contact sites and a significant 26.1% increase in the inner membrane fraction. In contrast, incubation with the C-28 StAR protein had no effect on cholesterol transfer. The cholesterol-transferring activity of the N-47 truncation, in contrast to that of the C-28 mutant, was corroborated when COS-1 cells were cotransfected with F2 vector (containing cytochrome P450 side-chain cleavage enzyme, ferridoxin, and ferridoxin reductase) and either pCMV empty vector or the complementary DNAs of wild-type StAR, N-47 StAR, or C-28 StAR. Pregnenolone production was significantly increased in both wild-type and N-47-transfected cells, whereas that in C-28-transfected cells was similar to the control value. Finally, immunolocalization studies with confocal image and electron microscopy were performed to determine the cellular location of StAR and its truncated forms in transfected COS-1 cells. The results showed that wild-type and most of the C-28 StAR protein were imported into the mitochondria, whereas most of N-47 protein remained in the cytosol. These studies demonstrate a direct effect of StAR protein on cholesterol transfer to the inner mitochondrial membrane, that StAR need not enter the mitochondria to produce this transfer, and the importance of the C-terminus of StAR in this process. (*Endocrinology* **139:** 3903–3912, 1998)

BIOSYNTHESIS of all steroids begins with the conversion of cholesterol to pregnenolone, as catalyzed by the cytochrome P450 side-chain cleavage enzyme (P450scc), which resides on the matrix side of the inner mitochondrial membrane (1, 2). Delivery of the substrate cholesterol to P450scc is a critical step because of the hydrophobic nature of cholesterol and the aqueous barrier present in the intermembrane space of mitochondria. To supply sufficient substrate to P450scc, it is necessary to overcome this barrier, a step that requires *de novo* protein synthesis (3–5). It has recently been proposed that the protein responsible for effecting the transfer of cholesterol to the inner mitochondrial membrane and thus acting as the regulatory protein in this process is the steroidogenic acute regulatory (StAR) protein (reviewed in Refs. 6–9). Many studies have shown that stimulation of various steroidogenic cells resulted in the increased synthesis of steroids and a concomitant increase in StAR expression (10–13). Also, it has recently been demonstrated that stimulation of bovine adrenal glomerulosa cells with Ca^{2+} increased StAR protein synthesis, cholesterol transfer to the inner mitochondrial membrane, and steroid production in a temporally related fashion (14, 15). However, definitive data indicating that the presence of StAR protein can directly result in the transfer of cholesterol to the inner mitochondrial membrane is still lacking, and little is known about the mechanism by which cholesterol transfer results from StAR expression.

Mutations in the *StAR* gene sequence of patients who suffer from the potentially lethal disease, lipoid congenital adrenal hyperplasia, indicated that the C-terminal portion of the StAR protein was important in cholesterol delivery and steroid biosynthesis (16). Further, it was demonstrated that constructs lacking the C-terminal region, but not those lacking the N-terminal region, of the StAR protein were unable to support steroid synthesis when transfected into COS-1 cells (17). Therefore, in an effort to demonstrate a direct link among specific regions of the StAR protein, cholesterol transfer, and steroid biosynthesis, we constructed

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N- and C-terminal truncations of StAR and examined their effects on cholesterol transfer and steroid production. In addition, we determined their cellular locations during the process of cholesterol transfer and steroid biosynthesis.

Materials and Methods

Chemicals

DMEM, kynurenine sulfate, aminoglutethimide, 22(*R*)-hydroxycholesterol, low gelling temperature agarose (A-4018), and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Waymouth's MB/752 medium, FBS, horse serum, Lipofectamine, trypsin-EDTA, antibiotics, and PBS were obtained from Life Technologies (Gaithersburg, MD, or Paisley, Scotland). [^{3}H]Cholesterol was purchased from DuPont-New England Nuclear (Boston, MA). Anti-StAR antisera against amino acids 88–98 of mouse StAR protein were produced in rabbits by Research Genetics (Huntsville, AL). Mouse monoclonal antibodies to cytochrome *c* oxidase subunit IV were purchased from Molecular Probes (Eugene, OR). All of the restriction enzymes were purchased from Promega (Madison, WI). Other common chemicals used in these studies were obtained from either Sigma or Fisher Chemicals (Fairlawn, NJ).

Cell culture

The COS-1 cell line was purchased from American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 10% FBS, penicillin G sodium (100 U/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (250 ng/ml). MA-10 mouse Leydig tumor cells were a generous gift from Dr. Mario Ascoli (Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA) and were grown in Waymouth's MB/752 medium containing 15% horse serum as previously described (18). Both COS-1 and MA-10 cells were incubated in 5% CO_2 at 37 C.

Plasmid construction of StAR truncations

To delete the first 47 amino acids at the N-terminus of the mouse StAR protein, PCR was used, and the methionine at position 48 was selected to initiate the N-47 truncated StAR mutant. The 5′-primer, 5′-CCA GAA TTC ACC ATG GGT CAA GTT CGA CG-3′, was designed to introduce an *Eco*RI restriction site. A *Hin*dIII restriction site was introduced 17 bp downstream of the stop codon (TAA) using the primer 5′-TAT AAG CTT AAT GTG GTG GAC AG-3′. PCR was performed for 30 cycles of 92 C for 1 min, 45 C for 30 sec, and 72 C for 30 sec, each cycle in a buffer containing 50 mM KCl; 10 mM Tris-HCl (pH 8.4); 0.1% Triton X-100; 2.5 mM MgCl; 0.2 mM each of deoxy (d)-ATP, dGTP, dTTP, and dCTP; 50 pmol of each primer; 5 U *Taq* DNA polymerase; and 0.5 μg pCMV5/StAR template. The PCR fragments were digested with *Eco*RI/*Hin*dIII and inserted into the pCMV plasmid (10) cut with the same enzymes.

For plasmid construction of the C-28 amino acid truncated StAR mutant, the 5′-primer, 5′-CCA GAA TTC GTC GAC CCA CGC GTC CGC-3′, was used to introduce an *Eco*RI restriction site immediately before the initiation codon (ATG) of the StAR complementary DNA (cDNA). The 3′-primer, 5′-ATA AAG CTT TTA GTT GAT GAT TGT CTT CGG-3′, was designed to introduce a *Hin*dIII restriction site and a stop codon at the position of the C-28 amino acid. The 858-bp fragments obtained from PCR were digested and inserted into *Eco*RI/*Hin*dIII sites of the pCMV plasmid using the same procedure as that used for the N-47 truncation.

Transfection of COS-1 cells

The procedure used for the transfection of COS-1 cells has been previously described (19). Briefly, plasmids of pCMV, pCMV/StAR, pCMV/N-47 StAR truncation (N-47), or pCMV/C-28 StAR truncation (C-28) were transiently transfected into COS-1 cells for a 6-h period using Lipofectamine (Life Technologies). Forty-eight hours after transfection, COS-1 cells were harvested and sonicated three times for 30 sec each time in import buffer containing 0.25 M sucrose, 10 mM MOPS buffer (pH 7.4 with KOH), 80 mM KCl, 5 mM MgCl, 3% BSA, 10 mM isocitrate, and 2 mM NADH. The sonicates were centrifuged at 600 × *g* to remove the debris, and the supernatants were retained.

Steroid production in COS-1 cells

COS-1 cells were transfected for 6 h with plasmids containing pCMV only, pCMV/StAR, pCMV/N-47, or pCMV/C-28 as well as with F2 plasmid (20). The cells were cultured for an additional 48 h, and the medium was collected. The cells were washed with PBS and cultured for an additional 2 h with 25 μM 22(*R*)-hydroxycholesterol to authenticate F2 transfection. The medium was collected, and samples from both the 48- and 2-h incubations were tested for pregnenolone production by RIA as previously described (19).

Cholesterol transfer in MA-10 mitochondria

MA-10 cells were cultured overnight in 100-mm plates containing 1 μCi [^{3}H]cholesterol/10 ml medium. The cells were collected, washed in cold PBS, then suspended in import buffer containing 0.76 mM aminoglutethimide to block metabolism of cholesterol by the P450scc enzyme. The cells were gently homogenized by hand using a glass on glass homogenizer. The MA-10 cell homogenates were divided into four 1-ml aliquots. Five hundred microliters of cell lysate from COS-1 cells transfected with pCMV, StAR, N-47, or C-28 were then added to each aliquot of MA-10 cell homogenate, mixed, and incubated for 2 h at 37 C. The samples were diluted with 5 ml cold Tris buffer containing 10 mM Tris (pH 7.4), 0.25 M sucrose, 1.0 mM EDTA, and 0.76 mM aminoglutethimide, and mitochondria were isolated as previously described (21).

Subfractionation of mitochondria

Submitochondrial membrane fractions were separated on sucrose gradients following a previously described procedure (14, 15, 22). Briefly, the mitochondrial pellet containing 2 mg protein was resuspended in cold 10 mM sodium phosphate buffer, pH 7.4, containing 0.76 mM aminoglutethimide and kept on ice for 20 min. Sucrose (1.8 M) was then added to give a final concentration of 0.45 M. After another 20-min period, the mitochondria were sonicated three times for 30 sec each time. The sonicated sample was centrifuged at 8000 × *g* for 15 min. The supernatant was collected and centrifuged at 150,000 × *g* for 90 min. The membrane-containing pellet was resuspended in 10 mM phosphate buffer, pH 7.4, containing 0.45 M sucrose and 0.76 mM aminoglutethimide. The membrane suspension was layered onto a linear sucrose gradient (15–50%) and centrifuged at 100,000 × *g* for 20 h at 4 C. Each gradient was collected into 20 fractions. The locations of mitochondrial membrane marker enzymes in this gradient were determined. Fractions 3–8 (corresponding to high activities of outer membrane-specific enzyme, kynurenine hydroxylase), 9–13 (containing both kynurenine hydroxylase and cytochrome *c* oxidase), and 14–18 (containing high amounts of cytochrome *c* oxidase, a inner membrane marker) were pooled, respectively. The pooled fractions were tested again to check the distribution of the marker enzymes. The amount of [^{3}H]cholesterol present in each fraction was assayed using a Beckman LS 6500 scintillation counter (Beckman, Fullerton, CA).

Marker enzyme assays

Kynurenine hydroxylase, an outer mitochondrial membrane enzyme, was assayed following Bandlow's procedure (23). The cytochrome *c* oxidase content of each fraction was determined by Western analysis using a monoclonal antibody against subunit IV and was quantitated using a BioImage Visage 2000 (BioImage Corp., Ann Arbor, MI) image analysis system, as previously described (21). The protein concentration was determined using a modification of the Bradford method (24).

Western blot analysis

The cell lysates from transfected COS-1 cells were tested for expression of StAR and its truncations by Western analysis as described previously (10). The samples were solubilized in sample buffer [25 mM Tris-HCl (pH 6.8), 1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue], boiled for 5 min, and loaded onto a 12% SDS-PAGE minigel (MiniProtean II System, Bio-Rad, Richmond, CA). Electrophoresis was performed at 200 V for 45 min using a standard SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3). The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad) at 100 V for 2 h at 4 C

using a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol, pH 8.3. The membrane was incubated in blocking buffer (PBS buffer containing 4% Carnation nonfat dry milk and 0.2% Tween-20) at room temperature for 1 h, followed by incubation with a primary antibody against StAR for 30 min. The membrane was washed with PBS containing 0.2% Tween-20 three times for 10 min each time. After incubation with the second antibody, donkey antirabbit IgG conjugated with horseradish peroxidase (Amersham, Arlington Heights, IL), the membrane was washed five times for 10 min each time. Specific protein bands were detected by chemiluminescence using the Renaissance Kit (DuPont-New England Nuclear, Wilmington, DE), and quantitated using the BioImage Visage 2000 (21).

Immunofluorescence staining and confocal microscopy analysis

For immunolocalization studies of wild-type and mutant StAR proteins, COS-1 cells (2×10^6 cells/0.8 ml) were transfected by electroporation (25) using 30 μg DNA StAR, N-47, or C-28. The electroporated cells were seeded onto 13-mm round glass slides and placed in wells of a 24-well plate (Nunc, Copenhagen, Denmark). After 48-h incubation in DMEM containing 5% FCS (Biological Industries, Kibbutz Beit-Haemek, Israel), the cell monolayers were further processed for immunofluorescence and confocal microscopy.

Fixation and immunofluorescence staining procedures used to visualize wild-type StAR and its mutants in cultured cells have been described previously (26). Incubations of permeabilized cells (26) with antisera to recombinant murine StAR (1:50) and lissamine-rhodamine-labeled goat (IgG) antirabbit IgG (1:20) were performed before confocal analysis of fluorescently labeled cells.

Confocal images were acquired using a confocal microscope (Bio-Rad MRC-1024 scanhead attached to a Zeiss Axiovert 135M). The cells were excited with the 514-nm line of an argon ion laser, and the emission was detected using an emission filter with a 580-nm center wavelength and a 32-nm band width. A $\times 63/(NA = 1.4)$ objective was used, and the iris aperture was 1–2 mm, resulting in optical sections between 1–2 μm. Intensity profiles were measured across the cells using Image Pro Plus (Media Cybernetics, Silver Spring, MD). First, a 3×3 median filter was applied to the images to remove point noise. Then, the average background value (as measured in an area in which cells were not present) was subtracted from the images. The long rectangle on each image denotes the area across which the profiles were measured. For each rectangle, the intensity was averaged across the short dimension of the rectangle, and these average values are shown as a function of distance along the long dimension of the rectangle.

Immunoelectron microscopy of COS-1 cells

Forty-eight hours after electroporation, COS-1 cells were harvested by brief trypsinization (2 min at 37 C) using commercial trypsin-EDTA solution (Biological Industries). Trypsinization was terminated by the addition of growth medium containing serum, and the cells were collected by centrifugation and fixed for 1 h at room temperature in a 0.05% electron microscopy grade glutaraldehyde solution (Electron Microscopy Sciences, Fort Washington, PA) in PBS (pH 7.4), freshly prepared with 3% paraformaldehyde (BDH, Poole, UK), 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$. After a brief washing with PBS, excessive aldehyde groups were neutralized by incubating the cells for 30 min in 0.1 M glycine in PBS. The cells were suspended in 50 ml melted agarose (low gelling temperature type VII) prepared in water (2%) and kept at 45 C. Cells were then pelleted by a brief centrifugation ($1000 \times g$), and the agarose was allowed to solidify at room temperature. Small cubes (1×1 mm) of agarose-embedded cells were dehydrated in graded dilutions of ethanol in water (30 min in 30%, 50%, and 70%) and embedded in LR White resin (London Resin Co., Bassingstoke, UK) as recently described (27). Thin sections were incubated with 1:20 dilutions of rabbit antisera to recombinant mouse StAR, followed by incubation with a 1:10 dilution of gold-labeled goat antirabbit IgG, as previously described (26). Epon-embedded cells were similarly fixed in 1% glutaraldehyde and 3% paraformaldehyde solution. After osmication, Epon blocks were prepared using standard procedures.

Statistical analysis

Each experiment was repeated at least three times. Statistical analyses of the data were performed using ANOVA and Duncan's multiple range test using the Statistical Analysis System (SAS Institute, Cary, NC)

Results

Characterization of submitochondrial membrane fractions

Membranes obtained from MA-10 cell mitochondria incubated with transfected COS-1 cell lysates were separated into different fractions by continuous sucrose density gradient centrifugation. The distribution of marker enzymes in the fractions indicates the presence of three distinct submitochondrial regions (Fig. 1). Near the top of the gradient in the least dense fractions (no. 3–8), a high level of the mitochondrial outer membrane specific marker enzyme, kynurenine hydroxylase (23), was found. In fractions close to the bottom of the tube, the more dense fractions (no. 14–18), a high concentration of cytochrome *c* oxidase, a mitochondrial inner membrane enzyme, was found. In addition to outer and inner mitochondrial membrane fractions, a third membrane population of intermediate density was found. Fractions 9–13, possessed both kynurenine hydroxylase and cytochrome *c* oxidase, characteristic of mitochondrial contact sites.

Intramitochondrial cholesterol transfer

Figure 2 demonstrates the expression of StAR and its truncations in COS-1 cells. Cells transfected with wild-type StAR cDNA contained high levels of the 37-kDa StAR precursor and the 30-kDa mature form as reported previously (19). N-47 and C-28 truncated proteins were also identified by Western blot analysis. Lysates from COS-1 cells transfected with cDNA of StAR or its truncations were incubated together with mitochondria from unstimulated MA-10 cells that had been preloaded with [^{3}H]cholesterol. After incubation, the mitochondria were collected, sonicated, and fractionated on sucrose gradients, and the various membrane fractions were pooled and assayed for [^{3}H]cholesterol content (Fig. 3). Incubation of the mitochondria with COS-1 cell lysate containing wild-type StAR protein resulted in a highly significant 34.9% increase in [^{3}H]cholesterol content in the contact sites ($P < 0.01$) and a 32.8% increase in the inner mitochondrial membranes ($P < 0.01$). Incubation with cell lysate containing N-47 protein increased the [^{3}H]cholesterol content of contact sites by 16.4% (not significant) and that of the inner membranes by 26.1% (highly significant, $P < 0.01$). The [^{3}H]cholesterol content in submitochondrial fractions from C-28-treated mitochondria did not show any differences compared with incubations with lysates from empty vector transfected cells. There was no significant difference in the [^{3}H]cholesterol content of outer membranes among all the treatments used in these studies.

Steroid production in transfected COS-1 cells

The results of steroid production are shown in Fig. 4. After cotransfection of COS-1 cells with the cDNA of wild-type StAR and the F2 plasmid, the pregnenolone concentration in the culture medium significantly increased from 161 ± 8 to

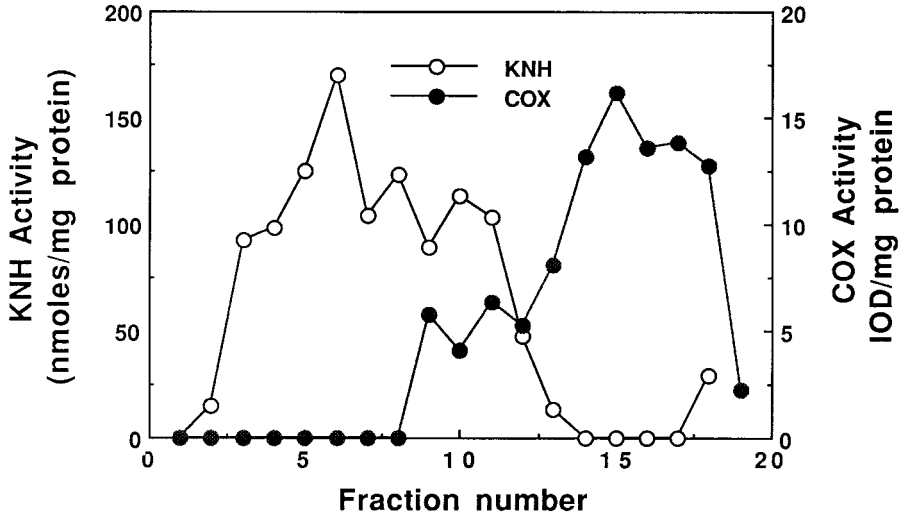

FIG. 1. Separation of submitochondrial membrane fractions on sucrose density gradients. Mitochondrial membranes suspended in 10 mM phosphate buffer, pH 7.4, containing 0.45 M sucrose were loaded onto a 15–50% linear sucrose gradient, centrifuged at 100,000 × *g* for 20 h, and collected into 20 fractions. Each fraction was analyzed for the activity of the outer mitochondrial membrane enzyme, kynurenine hydroxylase (KNH), which is expressed as nanometers of hydroxykynurenine formed per mg protein. Western analysis of the inner mitochondrial membrane protein, cytochrome *c* oxidase (COX), was performed (not shown) and expressed as integrated optical density (IOD) per mg protein in each fraction, also as shown. As a result of these assays, fractions 3–8 (containing high activities of KNH) were designated OM (outer membrane), fractions 9–13 (containing both KNH and COX) were designated CS (contact sites), and fractions 14–18 (containing a high content of COX) were designated IM (inner membrane).

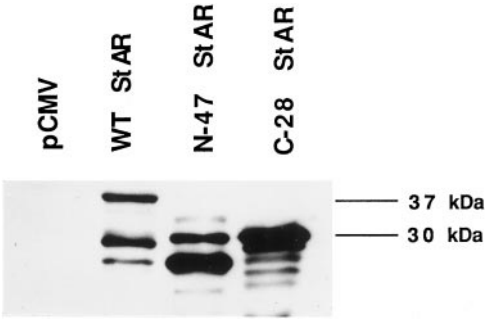

FIG. 2. Western blot analysis of StAR and its truncations expressed in transfected COS-1 cells. COS-1 cells were transfected with plasmids of pCMV, pCMV/StAR, pCMV/N-47, or pCMV/C-28 using Lipofectamine. Transfection was carried out for 6 h, followed by a 48-h incubation, at which time the COS-1 cell lysate was collected as described in *Materials and Methods* and prepared for gel electrophoresis. After electrophoresis, all samples were analyzed for StAR content using Western analysis with a polyclonal antisera to StAR protein as described in *Materials and Methods*. Lane 1, pCMV; lane 2, pCMV/StAR; lane 3, pCMV/N-47; lane 4, pCMV/C-28.

2013 ± 95 pg/ml (Fig. 4). Cotransfection with the N-47 truncation resulted in a similar increase (1691 ± 79 pg/ml). However, in the C-28 group, the pregnenolone concentration in the culture medium was only 262 ± 12 pg/ml, similar to that in controls. To test the transfection efficiency and activity of the P450scc introduced by transfection with F2 plasmid, 22(*R*)-hydroxycholesterol was added as a substrate. There was no significant difference in pregnenolone production among the groups after the addition of 22(*R*)-hydroxycholesterol (Fig. 4).

Confocal and immunoelectron microscopy

Figure 5, A–C, shows the results of immunofluorescence staining and confocal microscopy analysis of StAR and its truncations expressed in COS-1 cells. Wild-type StAR protein was readily imported into mitochondria, as reported previously (19), and showed a distinct mitochondrial localization. In Fig. 5A, StAR" (*left*) depicts a low power conventional fluorescence image of wild-type StAR. It shows a typical staining of filamentous mitochondria (*inset*). As expected, only 15–20% of the cells in a typical transfection experiment expressed StAR, whereas nonexpressing cells showed background diffuse staining. The same result was shown by confocal image, shown in the *middle panel* (Fig. 5A, StAR). The relative fluorescence intensities from quantitative analysis of this image (see *right panel*) also indicated that the majority of the StAR protein was localized in mitochondria. Figure 5B shows the distribution of the N-47 protein in COS-1 cells, which is quite different from that of wild-type StAR. The results from both the confocal image (*left panel*) and quantitative analysis of the confocal image (*right panel*) demonstrated the high distribution of this truncated protein in the cytosol and nucleus. No mitochondrial fluorescence signals could be detected that were above the cytosolic labeling intensities. We also performed ultrastructural localization of N-47 expression by immunoelectron microscopy. Figure 6 shows that, indeed, the vast majority of the immunogold particles decorating the N-47 antigenic sites remained in the cytosol, whereas the inner compartments of the mitochondria were practically devoid of labeling. A control study of COS-1 cells transfected with wild-type StAR (Fig. 6B) showed the reverse distribution of StAR, in which over 95% of the gold particles were inside the mitochondria. It is noteworthy that in N-47 transfected cells, a substantial number of the gold particles resided on the outer mitochondrial membrane (Fig. 6A). Also, in some of the cells expressing high levels of the transfected construct, a massive concen-

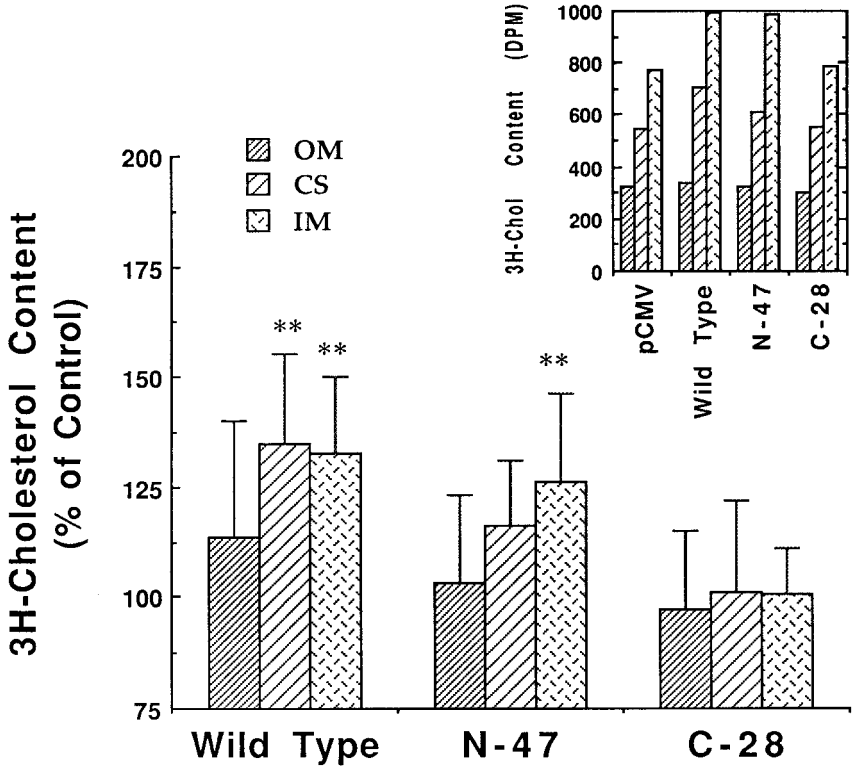

FIG. 3. The effect of StAR protein and its truncations on [^{3}H]cholesterol transfer in MA-10 cell mitochondria. MA-10 cells were incubated for 16 h with 1 μCi [^{3}H]cholesterol/10 ml medium. Cells were homogenized, and the cell homogenate, containing 0.76 mM aminoglutethimide, was incubated for 2 h with cell lysate from COS-1 cells transfected with the cDNA pCMV empty vector (control), pCMV/wild-type StAR, pCMV/N-47, or pCMV/C-28. The mitochondria were collected after incubation. Mitochondrial membranes were prepared and separated into outer membrane (OM), contact site (CS), and inner membrane (IM) on sucrose density gradients as described, and each fraction was assayed for [^{3}H]cholesterol content. The [^{3}H]cholesterol contents (in disintegrations per min) of OM, CS, and IM are shown in the *inset*. The amounts of [^{3}H]cholesterol in OM, CS, and IM treated with wild-type StAR-, N-47-, or C-28-containing lysate were then expressed as a percentage of that found using control lysate. **, Highly significant ($P < 0.01$; n = 6).

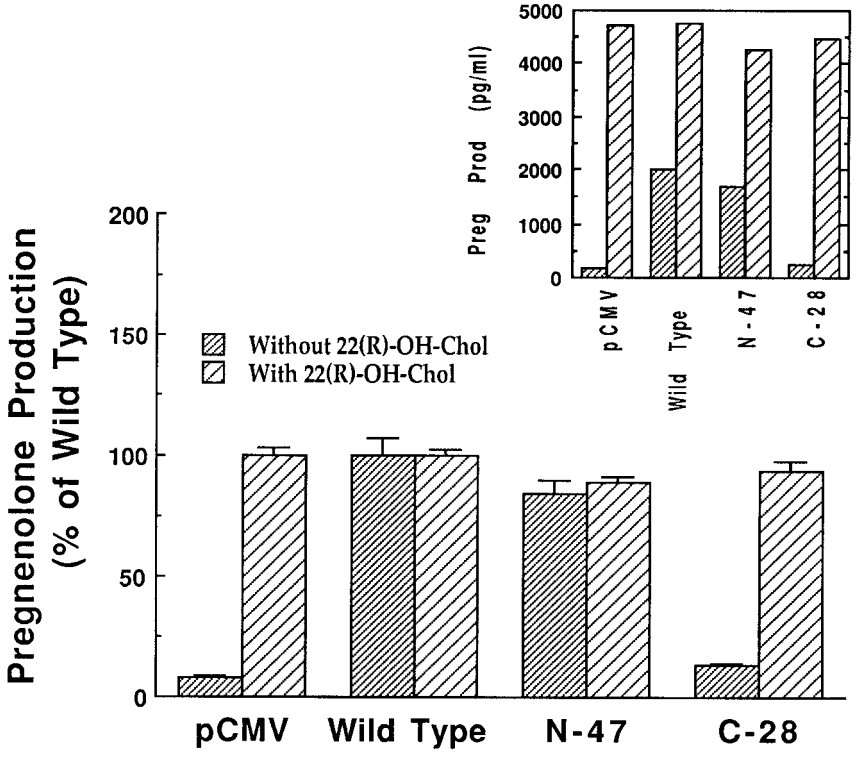

FIG. 4. The effect of StAR protein and its truncations on steroid production in COS-1 cells. COS-1 cells were transfected with plasmids containing pCMV empty vector, pCMV/StAR, pCMV/N-47, or pCMV/C-28 and then cotransfected with F2 plasmid (a vector containing P450scc, adrenodoxin, and adrenodoxin reductase) for each group. The cells were cultured for 48 h, and the medium was collected. The cells then were washed with PBS and cultured for an additional 2 h with 25 μM 22(R)-hydroxycholesterol. The media from the 48- and 2-h incubations were collected and assayed for pregnenolone production by RIA. The absolute amount of pregnenolone production is shown in the *inset*, and the values obtained were then expressed as a percentage of wild-type steroid production.

tration of N-47 StAR antigen was noticed in electron-dense lysosome-like bodies (Fig. 6A′).

Localization of the C-28 protein in COS-1 cells is shown in Fig. 5C. The confocal image (*left panel*) shows exclusive staining of the mitochondria, most of which are circular in shape. Quantitative analysis also indicated that most of the C-28 protein was localized in mitochondria. To better understand the unusual shape of the C-28 transfectants, immunoelectron microscopy and standard transmission electron microscopy were conducted to reveal the ultrastructural details of the

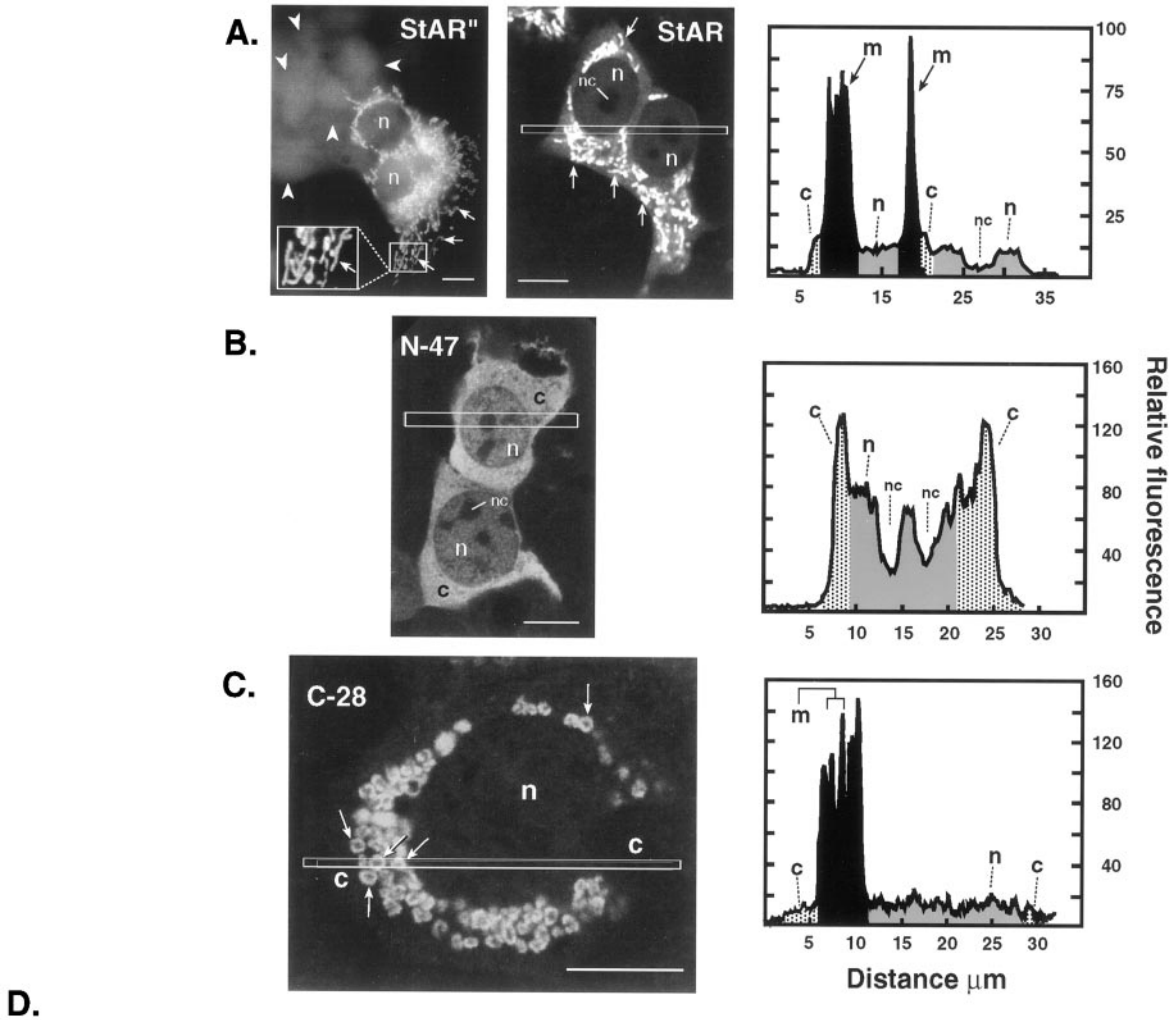

FIG. 5. Immunolocalization of StAR mutants expressed in COS-1 cells. COS-1 cells were transfected with different StAR cDNA constructs, and 48 h later, the cellular localization of StAR was studied by immunofluorescence analysis as described in *Materials and Methods*. Confocal images (*left panels*) were acquired using a confocal microscope, and the fluorescence intensity profiles across the long rectangle denoted in each image were measured as described in *Materials and Methods*. The *right panels* present the average fluorescence intensity values across the cells as a function of distance along the long dimension of the rectangle. A, StAR" (*left*) depicts a low power conventional fluorescence image of cells expressing wild-type

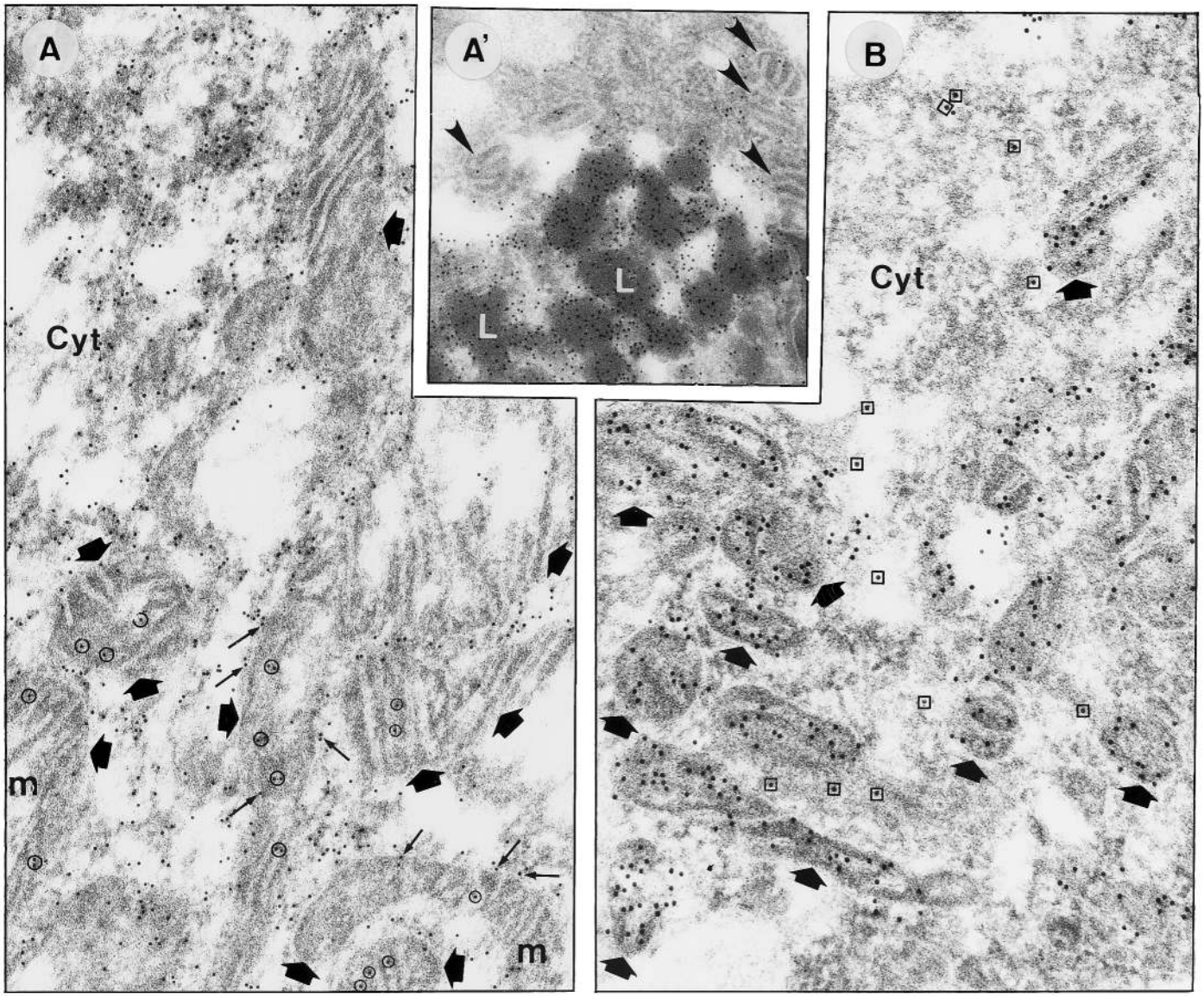

FIG. 6. Ultrastructural localization of N-47 and wild-type StAR expressed in COS-1 cells. COS-1 cells were transfected with N-47 StAR cDNA construct (A) or wild type StAR as a control (B). Forty-eight hours later, the cells were harvested and prepared for immunogold electron microscopy as described in *Materials and Methods*. A, Note that the vast majority of N-47 StAR gold labeling localizes to the cytosol (Cyt), and fewer particles (denoted by *circles*) are observed inside the mitochondria (m). Particles located on the outer membranes of the mitochondria are denoted by *arrows*. Magnification, ×50,600. A′, Lysosome-like electron dense bodies heavily labeled with StAR antibody are frequently seen in N-47-expressing cells. Note that the size of each vesicle (L) matches the size of the mitochondria (*arrowheads*). Magnification, ×40,400. B, Note ample gold labeling of wild-type StAR inside the mitochondria (*thick arrows*). By contrast, very few gold particles (denoted by *squares*) are localized in the cytosol (Cyt). Magnification: c, ×61,000.

StAR. Note the typical staining of filamentous mitochondria (*inset*) denoted by *arrows*. Diffuse background staining of nontransfected cells (*arrowheads*) represents a similar labeling pattern of cells transfected with empty pCMV vector (not shown). StAR (*middle panel*) presents a confocal image depicting two cells (fused as a result of electroporation) expressing wild-type StAR. Quantitative analysis of this image provides the relative fluorescence intensities (*right panel*) in the mitochondria (m, *black*), the cytosol (c, *dotted*), and the nuclear area (n, *gray*). nc, Nucleolus. B, Confocal image of two cells expressing the N-47 deletion construct of StAR. Image analysis was conducted as described for StAR above. Note the high cytosolic and nuclear staining. C, Confocal image of a single cell expressing the C-28 deletion construct of StAR. Image analysis was conducted as described for StAR above. Note the exclusive staining of the mitochondria, most of which are circular in shape (*arrows*). *Bars* represent 10 mm. D, a, High power electron micrograph depicting two circular doughnut-shaped mitochondria (*thick arrows*) in C-28-expressing cells. Note the gold labeling (*thin arrows*) of C-28 StAR in the mitochondria and an apparently "white" cavity, free of electron-dense material (*), which confers a doughnut shape to the immunofluorescently labeled mitochondria seen in C. Note that the cytoplasm (Cyt) is free of gold labeling. D, b and c, The doughnut-shaped mitochondria could also result from a curving process of an elongated mitochondrion to form a closed circle, as demonstrated by *arrows* in b (Epon-embedded cells) and c (LR White-embedded cells). Note that this process creates a central mitochondrial cavity that is filled with electron-dense cytoplasm (#) entrapped by the fusing ends of the circular mitochondrion (*arrowheads* in c). Magnifications: a, ×50,600; b, ×40,400; c, ×61,000.

aberrant mitochondria. Figure 5D shows that either one of two processes conferred a circular doughnut shape to the mitochondria harboring the C-28 truncated StAR; Fig. 5Da shows that the C-28-containing mitochondria are devoid of electron-dense material in their center, as if the cristae membranes collapsed to form an apparently empty hole in the mitochondrial core. Osmicated sections of such mitochondria (not shown) did not reveal any membrane-rich structures, as has been documented in mitochondria of cells undergoing necrosis (28). Alternatively, Fig. 5D, b and c, depicting C-28 transfected cells, show a process for curving of an elongated mitochondria to form a closed circle. Therefore, the circular mitochondria appear to engulf the electron-dense cytoplasm observed in the center portion of the organelles (indicated by #).

Discussion

For steroid hormones to be synthesized, the precursor for all steroids, cholesterol, must be transported to the inner mitochondrial membrane where P450scc, which converts it to pregnenolone, resides. As cholesterol is very hydrophobic, and its diffusion through water is very slow (29, 30), it cannot traverse the aqueous intermembrane space within a time frame consistent with the rate of steroid hormone biosynthesis known to occur in response to trophic hormone stimulation. Thus, the delivery of cholesterol requires some assistance if it is to reach the P450scc enzyme rapidly, and it has long been known that this step requires the *de novo* synthesis of a protein whose role is believed to be the regulation of this transfer (31–33). A protein known as the StAR protein has been shown to be a strong candidate in fulfilling the role of this regulatory protein. The case for StAR as the acute regulator has been made recently in several review articles (6–9).

Previous studies have indicated that *de novo* synthesis of StAR protein resulted in steroid production. However, a direct link between the presence of StAR protein and cholesterol transfer had not been made. Three lines of evidence obtained in the present study directly demonstrated the effects of StAR protein on intramitochondrial cholesterol transfer. 1) After incubation of MA-10 mitochondria with StAR-containing cell lysates, the [^{3}H]cholesterol contents of contact sites and inner mitochondrial membrane fractions increased significantly over those in controls containing no StAR in the lysates. These results are in agreement with previous studies that indicated that stimulation of bovine adrenal glomerulosa cells with the steroidogenic stimuli angiotensin II or Ca^{2+} resulted in an increased content of cholesterol in both contact sites and the inner mitochondrial membrane (14, 15). 2) The increase in cholesterol transfer was directly correlated with an increase in steroid synthesis when COS-1 cells were cotransfected with F2 plasmid and wild type StAR. 3) The lack of steroid production was also correlated with the inability of a C-terminus truncated StAR protein to mobilize [^{3}H]cholesterol into the contact sites and inner membranes. Conversely, the N-47 truncation mutant of StAR was capable of conferring both high steroidogenic activity and cholesterol transfer to the inner membranes of the mitochondria. The magnitude of the cholesterol transfer observed in the present study in response to wild-type and N-47 StAR is similar to that previously observed in Ca^{+2}-clamped bovine adrenal glomerulosa cells (14, 15).

Very little is known concerning the mechanism by which StAR protein is involved in cholesterol transfer to the mitochondrial inner membrane. Evidence from patients with lipoid congenital adrenal hyperplasia strongly supports the importance of the C-terminal region of the StAR protein in supporting steroidogenesis (16, 34, 35). One patient in particular had a mutation in the StAR gene that resulted in the insertion of a stop codon that removed the C-terminal 28 amino acids from the wild-type StAR protein (16). The results from the present study shows that the C-terminus of StAR is indeed critical in cholesterol transfer, clearly illustrating that the C-28 truncation was unable to transfer cholesterol to the inner mitochondrial membrane in isolated MA-10 cell mitochondria. As further corroboration, expression of this StAR cDNA in COS-1 cells indicated that the protein was completely inactive in promoting steroidogenesis. Similar results were obtained using mutated cDNAs from lipoid CAH patients in which expression of C-terminal truncated forms of the StAR protein in COS-1 cells resulted in either partially diminished steroidogenic capacity when 10 amino acids were removed or completely diminished capacity when 25 amino acids were removed (17).

No less curious were the results obtained with the N-47 mutant of StAR. Very little of this truncated form of StAR lacking its signal peptide entered the mitochondria, as indicated by Western blot analyses. Confocal microscopy and ultrastructural visualization studies confirmed that ample amounts of the N-47 truncated protein resided in the cytosol, whereas the inner compartments of the mitochondria were devoid of the antigen. Yet, a substantial, but not exclusive, immunogold decoration of N-47 on the surface of the outer mitochondrial membrane could suggest that truncation of the amino-terminus still allowed a potential interaction of N-47 StAR with the organelle, even in the visualized absence of its import. Such interaction may explain the fact that the N-47 truncation is fully active in both cholesterol transfer and stimulation of steroidogenesis.

Previously we have employed an affinity column constructed of a synthetic signal sequence of the StAR protein and have used it to purify a mitochondrial protein complex that showed specific binding to a ^{35}S-labeled signal peptide (36). Although the identities of the components of this complex remain unknown, it is highly likely that the N-terminal sequence of StAR binds to the mitochondrial translocase machinery, which facilitates the import of the StAR precursor into the organelle. As the N-47 truncation is fully active biologically, whereas the transfected C-28 can neither augment steroidogenesis nor facilitate cholesterol transfer, these observations provide further evidence for the importance of the C-terminus for the function of StAR. This finding also raises the question of the possible function of the import process in steroidogenesis. An early model from our laboratory hypothesized that cholesterol transfer may occur as a result of the import and processing of the StAR protein into the mitochondria (6, 7). This model was based on the well characterized observation that import of mitochondrial proteins resulted in the formation of contact sites between the outer and inner mitochondrial membranes at the point of

insertion of the protein (37–39). Therefore, it was reasoned that perhaps during the formation of these contact sites, cholesterol would be able to transfer to the inner mitochondrial membrane, as the aqueous intermembrane barrier was essentially removed (40). This model predicted that it was the insertion of the N-terminus of the StAR protein that would be important for cholesterol transfer. At this time, however, the majority of observations indicate that import of the StAR protein into the mitochondria is not required for its ability to stimulate steroidogenesis (16, 17). Thus, as indicated previously, perhaps the import of the StAR protein functions as an off switch by removing the protein from the outer membrane where its function is expressed (17).

Like wild-type StAR, the C-28 truncated form was almost totally imported into the mitochondria, as readily observed with confocal microscopy analyses. Curiously, there were morphological differences in the shape of the mitochondria after import of the C-28 form compared with wild-type StAR import. Import of the C-28 protein resulted in the formation of doughnut-shaped mitochondria as observed in the immunofluorescence studies. This alteration in morphology was confirmed in the results obtained from immunoelectron microscopy, which showed a curving process of elongated mitochondria to form closed circular organelles. The reasons for these morphological differences and their possible consequences are unknown at this time. Perhaps the removal of the C-terminal portion of the StAR protein affected the manner in which the protein is refolded after import into mitochondria, resulting in an improper conformation that caused the curving process.

Recent studies have shown that in addition to an increase in their cholesterol content, the contact sites and inner mitochondrial membranes of Ca^{2+}-stimulated bovine adrenal glomerulosa cells also contained StAR protein and the first two enzymes in the steroidogenic pathway, namely P450scc and 3β-hydroxysteroid dehydrogenase (15, 22, 41). Therefore, the colocalization of these three proteins raises the possibility that StAR initiates the formation of a protein complex that could result in cholesterol transfer, then convert cholesterol directly into progesterone. This complex may contain 3β-hydroxysteroid dehydrogenase and P450scc, as alluded to above. In addition, based on the observations of Papadopoulos and colleagues (42–45), it is possible that if such a complex exists, it may include the peripheral benzodiazepine receptor and its ligand the diazepam binding inhibitor proteins. Interestingly, the possible existence of a multiprotein complex in steroidogenic mitochondria, referred to as hormonads, was predicted several years ago by Lieberman and colleagues (46, 47). It will be of interest to determine whether an association between any or all of these proteins exists in the mitochondrial membrane and, perhaps even more interestingly, whether they exist in contact sites and if this association occurs preferentially with the C-terminal portion of the StAR protein.

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