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Steroidogenic Activity of StAR Requires Contact with Mitochondrial VDAC1 and Phosphate Carrier Protein*

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The steroidogenic acute regulatory protein (StAR) is required for adrenal and gonadal steroidogenesis and for male sexual differentiation. StAR acts on the outer mitochondrial membrane (OMM) to facilitate movement of cholesterol from the OMM to the inner mitochondrial membrane to be converted to pregnenolone, the precursor of all steroid hormones. The mechanisms of the action of StAR remain unclear; the peripheral benzodiazepine receptor, an OMM protein, appears to be involved, but the identity of OMM proteins that interact with StAR remain unknown. Here we demonstrate that phosphorylated StAR interacts with voltage-dependent anion channel 1 (VDAC1) on the OMM, which then facilitates processing of the 37-kDa phospho-StAR to the 32-kDa intermediate. In the absence of VDAC1, phospho-StAR is degraded by cysteine proteases prior to mitochondrial import. Phosphorylation of StAR by protein kinase A requires phosphate carrier protein on the OMM, which appears to interact with StAR before it interacts with VDAC1. VDAC1 and phosphate carrier protein are the first OMM proteins shown to contact StAR.

The steroidogenic acute regulatory protein (StAR),[2] a nuclear-encoded, mitochondrially targeted protein, is required for adrenal and gonadal steroidogenesis and for male sexual differentiation. StAR moves cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) where it is converted to pregnenolone, the precursor of all steroid hormones (Fig. 1*A*). Lack of StAR causes potentially lethal congenital lipoid adrenal hyperplasia (lipoid CAH) (1). StAR, which acts exclusively on the OMM (2, 3), is expressed in the cytoplasm as a 37-kDa protein, is activated by phosphorylation of Ser^{195} (4), processed to a 32-kDa intermediate, imported into mitochondria, and cleaved to a 30-kDa protein (5).

Despite intense investigation, the mechanisms of the action of StAR on the OMM remain unknown. Several models have been proposed to explain the mechanism of StAR action (6). Much attention has focused on the peripheral benzodiazepine receptor (PBR), an OMM protein that appears to be involved in steroidogenesis (7), but despite evidence for a functional interaction between PBR and StAR (8), the identity of the OMM proteins with which StAR interacts remains undefined. Here we demonstrate that StAR interacts with voltage-dependent anion channel 1 (VDAC1), and with phosphate carrier protein (PCP) on the OMM to initiate the action of StAR. VDAC1 and PCP are the first OMM proteins definitively shown to interact with StAR.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies against VDAC1, PCP, glyceraldehyde-3-phosphate dehydrogenase, PBR and COX IV were from Chemicon, Sigma, Calbiochem, and Santa Cruz Biotechnology, respectively. The generation of antiserum against bacterially expressed human StAR, which detects both phospho- and dephospho-StAR, has been described (9). Antiserum specific for phospho-StAR was a gift from Dr. Steven King (10, 11). Rabbit IgG was from Sigma, protein A beads were from Amersham Biosciences, and 2,4-dinitrophenol from Riedel-de Haen (Germany). Dithiocyanate was from Serva/Invitrogen, and 4-sulfophenyl isothiocyanate was from Research Organics. RNase A and Proteinase K were from Roche and GE Healthcare, and digitonin was from Calbiochem. Oligonucleotide primers were obtained from Integrated DNA Technology (Ames, IA). All other chemicals were purchased from Sigma, unless otherwise specified.

Plasmid Construction, Cell Culture, Transfection, Mitochondrial Isolation, and Western Blotting—Construction of full-length StAR, R182L StAR, P450scc, and SCC/N-62 StAR cDNA expression vectors was described elsewhere (3). For the StAR-DHFR fusion, residues 1 to 284 of StAR were fused to residues 2 to 224 of DHFR deleting the StAR stop codon and cloned in SP6 vector (3). Cell culture, transfections, and mitochondrial isolation from cultured cells were performed as described (3). In all cases 100 ng/ml trilostane (Steraloids, Los Angeles, CA) was added as an inhibitor of 3β-hydroxysteroid dehydrogenase. Media were collected from cultures 48 h after transfection and assayed for pregnenolone by radioimmunoassay (MP Biomedi-

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[2] The abbreviations used are: StAR, steroidogenic acute regulatory protein; OMM, outer mitochondrial membrane; IMM, inner membrane; VDAC1, voltage-dependent anion channel 1; PCP, phosphate carrier protein; PBR, peripheral benzodiazepine receptor; MS, mass spectrometry; KPA, Köenig's polyanion; PITC, phenylisothiocyanate; siRNA, small interfering RNA; DHFR, dihydrofolate reductase; ANT, adenine nucleotide transporter; PKA, protein kinase A; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl) propane-1,3-diol.

cals) as described (1). For Western blot analysis, cells were lysed in 1× SDS sample buffer (0.1% SDS, 10 mM Tris, pH 6.8, 100 mM β-mercaptoethanol or 10 mM dithiothreitol), resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were incubated in 1:100 dilutions of primary antibody, with the exception of anti-StAR antiserum (1:10,000) and anti-phosphoserine StAR antiserum (1:800).

For mitochondrial isolation from adrenal tissue, sheep adrenals were isolated immediately after sacrifice. Adrenal tissue was placed in pre-chilled mitochondria isolation buffer (250 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 40 μg/ml protease mixture (Roche)). Tissue was then homogenized in an all glass, hand-held Dounce homogenizer with 12 up and down strokes, and cleared of debris by centrifugation at 3,500 × *g* for 10 min at 4 °C. The supernatant (mitochondrial fraction) was centrifuged at 10,000 × *g* at 4 °C, and the resulting mitochondrial pellet was washed twice and re-pelleted at 3,500 × *g*. For storage, pellets were suspended in import buffer (125 mM sucrose, 2 mM ATP, 2 mM NADH, 50 mM KCl, 0.05 mM ADP, 2 mM dithiothreitol, 5 mM sodium succinate, 2 mM $Mg(OAc)_2$, 2 mM KH_2PO_4, 10 mM creatine phosphate, 10 μl of creatine kinase (4 mg/ml), 10 mM HEPES pH 7.4), and frozen in liquid N_2 for *in vitro* bioactivity and protein import (12–14).

Chemical Cross-linking—Disuccinimidyl suberate (Pierce) was dissolved in Me_2SO and sequentially diluted with import buffer just before cross-linking. Freshly isolated mitochondria from adrenal tissues (100 μg) were incubated with cell-free synthesized [^{35}S]StAR for 15 min at 20 °C. Disuccinimidyl suberate was then added directly to the reaction mixture, to a final concentration of 0.1 mM, and incubated at 4 °C and at room temperature for 5 min to 2 h. The reaction was terminated by the addition of 10 mM Tris, pH 9.5, and 1× SDS sample buffer. Samples were then boiled for 15 min and analyzed by 12.5% SDS-PAGE. The darker radioactive band was excised and analyzed by mass spectrometry. Equivalent results were obtained with three other homobifunctional cross-linking agents: disuccinimidyl glutarate, bis(sulfosuccinimidyl)suberate, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

Mass Spectrometry—Excised bands were processed for mass spectrometry in an automated in-gel tryptic digestion ("Waters MassPREP Station" system, Waters, MA). Following electrophoresis, blue native-PAGE gels were fixed in 10% acetic acid, washed with water for 24 h, dried, and exposed to x-ray film. The radioactive gel bands were excised, reduced, alkylated with iodoacetamide, and digested with trypsin (Promega Sequencing grade modified). The resulting peptides were analyzed via liquid chromatography-mass spectrometry (MS)/MS (Waters) coupled with a Q-ToF-2 mass spectrometer (Micromass, UK/Waters, MA). Proteins were identified from MS/MS spectra by searching the NCBI non-redundant data base using Mascot MS/MS Ion Search (Matrix Science, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine, and one missed cleavage per peptide. In our experiment, an individual ion score greater than 39 (−10 log(P) at 95% confidence) indicated extensive homology; mass spectrometry scores ranged from 60 to 438.

Immunoprecipitation—Total digitonin extracts or fractions from sucrose density gradients were immunoprecipitated overnight at 4 °C with antibodies in 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 200 mM NaCl, 0.5% sodium deoxycholate, and protease inhibitor mixture. The protein-IgG complex was harvested with protein A-Sepharose CL-4B, eluted by boiling for 15 min in 1× SDS-sample buffer, and analyzed by SDS-PAGE.

Köenig's Polyanion Treatment—Köenig's polyanion (15) (KPA, a kind gift from Dr. Marco Colombini, University of Maryland) was dissolved in 10 mM NaOH, adjusted to pH 7.0 with 50 mM NaOAc, pH 4.0, and stored at 4 °C. Prior to use, the solution was equilibrated with import buffer or media as required. Two different concentrations of KPA (30 and 90 mg/ml) were incubated with COS-1 cells 24 h prior to co-transfection with StAR and the F2 fusion of the cholesterol side chain cleavage system (H_2N-P450scc-AdRed-Adx-COOH) (16). Pregnenolone concentrations in the media were then determined, and cell lysates were analyzed for StAR processing by Western blot. Western blot signals were developed by West-Pico chemilumiscent reagent (Pierce). For direct measurement of activity, mitochondria (2 μg of protein) were incubated with the above mentioned concentration of KPA for 2 h and then incubated with biosynthetic StAR. For transient VDAC channel blockage, mitochondria were incubated with 30 mg/ml KPA, re-isolated, and resuspended in import buffer. For analysis of StAR mitochondrial import, 100 μg of mitochondria were incubated with cell-free synthesized [^{35}S]StAR in the presence of 10 to 90 mg/ml KPA. Mitochondrial [^{35}S]StAR was then analyzed by SDS-PAGE.

Blocking Phosphate Carrier Protein—Mitochondria (20 mg of protein) isolated from adrenal tissues were incubated with 2 mM phenylisothiocyanate (PITC) or sulfo-PITC for 10 min on ice, and reactions were terminated by adding 10 mM dithiocyanate. Mitochondria were then washed twice with 10 mM dithiocyanate, resuspended in import buffer, and incubated with cell-free synthesized [^{35}S]StAR for 2 h at 26 °C. In some cases, 1 mM ATP was included in the reaction mixture. Mitochondrial import was examined by analyzing a 20-μl aliquot by SDS-PAGE.

Antibody Shift Native-PAGE and Blocking Experiments—Cell-free synthesized [^{35}S]StAR was imported into mitochondria (20 μg of protein) from MA-10 cells or sheep adrenals for 10 min. Mitochondria were then resuspended in 100 μl of mitochondria isolation buffer and incubated with antiserum (suspended in 40 μl of import buffer) for 30 min on ice. Reisolated mitochondria were washed with import buffer two times and extracted in digitonin buffer (1% digitonin) and analyzed by native-PAGE (17). For antibody blocking experiments, mitochondria were incubated with IgG or VDAC1 antiserum to VDAC1 prior to import.

Bioactivity in Isolated Mitochondria—Mitochondria (2 μg of protein) from control or VDAC1 knockdown MA-10 cells or from adrenal tissues were resuspended in a final volume of 100 μl of bioassay buffer (125 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM NaH_2PO_4, 10 mM isocitrate, 25 mM HEPES, 0.1 mM ATP, and 10 mg/ml cholesterol, pH 7.4), incubated with full-length, cell-free synthesized or biosynthetic StAR under the conditions

described (3). Supernatants were then assayed for pregnenolone by radioimmunoassay (1).

Generation of VDAC1 Antiserum—Antiserum specific to VDAC1 was custom raised by Alpha Diagnostic International (San Antonio, TX) against a keyhole limpet hemocyanin-conjugated synthetic peptide comprising amino acids 151–165. New Zealand White rabbits received an initial injection of keyhole limpet hemocyanin-conjugated peptide and 14 days later received an additional injection, followed by a booster injection on every 7th day to day 63. At this time, the antiserum titer exceeded 1:1000, as determined by enzyme-linked immunosorbent assay. Rabbits were then sacrificed and all serum was collected. The affinity purification of VDAC1 antibody was carried out after cross-linking a peptide corresponding to residues 151–165 of VDAC1 with a SulfoLink column following the manufacturer's instructions (Pierce, Sulfolink Immobilization kit). The SulfoLink column was equilibrated at room temperature with coupling buffer. Peptide-(151–165) (1 mg) was dissolved in coupling buffer, then coupled with the resin by mixing 15 min at room temperature on a rocker followed by an additional 30 min of incubation without rocking. The unbound peptide was removed by centrifugation and three washes with coupling buffer, then nonspecific sites of the column were blocked with 50 mM L-cysteine. Before purification, the column was equilibrated with phosphate-buffered saline. The antiserum was purified in batches. Antiserum (2 ml) was incubated with the column on a rocker for 30 min, the unbound antibodies were removed, and the column was washed three times with phosphate-buffered saline containing 100 mM NaCl and eluted with 100 mM glycine at pH 3.0, which was immediately titrated by addition of 65 μl of 1.0 M Tris, pH 9.5. The purified antiserum was dialyzed with phosphate-buffered saline.

VDAC1 Knockdown and Development of Stable Cell Lines—siRNA (CCCUAAAACCAGGUAUCAA) for VDAC1 was obtained from Ambion (Austin, TX). COS-1 and MA-10 cells were transfected with 30 and 60 pmol of VDAC1 siRNA, respectively, using Oligofectamine (Invitrogen), according to the manufacturer's protocol. Non-targeting siRNA (Ambion) served as a control in all experiments. A combination of two non-targeting scrambled siRNAs (Ambion) was also included as control in all experiments. A subset of cells was co-transfected with an expression vector for VDAC1 cDNA in which two nucleotides in the siRNA target region were mutated. VDAC1 was mutated without altering the amino acid sequence as follows: CCCTAAAA(C→T)CA(G→A)GTATCAA. The pSilencer siRNA expression plasmid was constructed by ligating the 55-mer siRNA template into a pSilencer 4.1 CMV expression vector (Ambion, TX). Complementary 55-mer siRNA oligonucleotides (5-GATCCCCCTAAAACCAGGTATCAATTCAAGAGATTGATACCTGGTTTTAGGGAGA-3′ and 3′-GGGGATTTTGGTCCATAGTTAAGTTCTCTAACTATGGACCAAAATCCCTCTTCGA-5′) were annealed, ligated into pSilencer neovector (Ambion), and transformed into competent *Escherichia coli* to screen for positive hairpin siRNA inserts. The accuracy of the knockdown was determined by Western blotting. For generation of stable cell lines, COS-1 and MA-10 cells were independently transfected with the purified plasmid using Lipofectamine (Invitrogen). Forty-eight hours after transfection, single clones were generated by limiting dilution into a selection medium containing 600 μg/ml G418 (Geneticin, GE Healthcare). Individual clones were then transferred to 24-well plates (Greiner, Germany) for propagation and then later transferred to 6-cm plates for transfection with StAR and F2. Individual clones were examined for VDAC1 expression by Western blotting. For knockdown of PCP, 20 pmol of PCP siRNA (GGUUAUGCCAACACUUUGATT) was transfected, as described for VDAC1 siRNA. Expression of PCP was examined 48 h after transfection. Western blotting was performed with 12.5 μg of protein and PCP-specific antibody (1:200) dilution. Signals were developed with West-Pico chemilumiscent reagent (Pierce).

In Vitro Transcription/Translation, Mitochondrial Import, and Protein Complex Isolation—In vitro translation was performed in the TNT rabbit reticulocyte system (Promega). Translation of cDNA was carried out in the presence of [^{35}S]methionine or unlabeled methionine with SP6 polymerase at 30 °C for 90 min, according to the manufacturer's instructions. Ribosomes and ribosome-bound nascent polypeptide chains were removed by ultracentrifugation at 148,000 × *g* for 20 min at 4 °C. Translated proteins were allowed to undergo brief import by incubation with sheep adrenal mitochondria (100 μg of protein) at 26 °C for 10 min. For import and blocking experiments, mitochondrial import of [^{35}S]StAR (2 μl) was allowed to proceed for 2 h at 26 °C (3). Partial proteolysis was carried out with 10 μg/ml proteinase K for 15 min at 4 °C. Reactions were terminated with phenylmethylsulfonyl fluoride and heat inactivation. For analysis of the assembled proteins in the complex, mitochondria were re-isolated and lysed in digitonin buffer ((1% (w/v) digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice (18). The digitonin lysate was combined with native-PAGE sample buffer (5% Coomassie Brilliant Blue G-250, 100 mM BisTris, pH 7.0, 500 mM 6-aminocaproic acid) and subjected to 3–16% gradient native-PAGE at 100 V overnight at 4 °C. Protein complexes were further fractionated by applying 100 μl of digitonin lysate to a 30-10% sucrose density step gradient, with a 200-μl 66% sucrose cushion at the bottom (final volume = 2.0 ml). Following centrifugation at 55,000 × *g* in a Beckman TLA55 rotor for 4 h, the sample was immediately equally aliquoted and loaded onto gradient, native-PAGE (20 μl) or SDS-PAGE gels (20 μl). Radiolabeled proteins were detected by digital autoradiography or on a phosphorimager.

Pulse-Chase Labeling Experiments—COS-1 cells transfected with StAR or N-62 StAR were incubated with methionine-free Dulbecco's modified Eagle's medium without serum for 30 min, then labeled with 0.1 mCi of [^{35}S]methionine for 30 min. After labeling, the medium was changed to Dulbecco's modified Eagle's medium containing 4 mM methionine and chased for the times indicated. Cells were than scraped into immunoprecipitation buffer containing 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 200 mM NaCl, 0.5% sodium deoxycholate, and protease inhibitor mixture. The lysed cells were

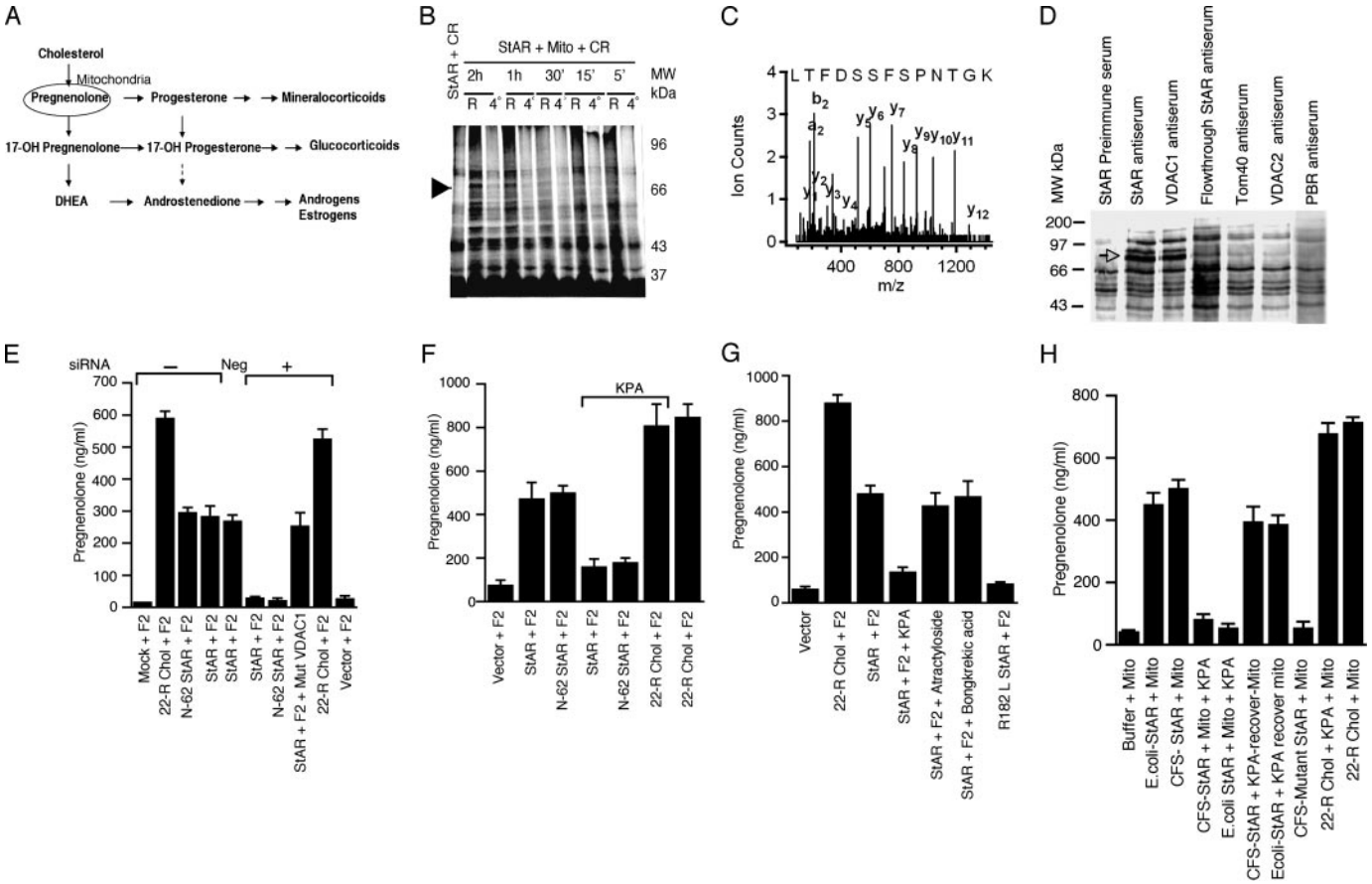

FIGURE 1. **Identification of the interaction of VDAC1 with StAR on the OMM.** *A*, simplified scheme of steroid hormone biosynthesis. *B*, disuccinimidyl suberate cross-linking (*CR*) of [^{35}S]StAR synthesized in a cell-free transcription/translation system to OMM proteins on fresh mitochondria at 4 °C and at room temperature (*R*). A 75-kDa band (*arrowhead*) appeared with increased cross-linking time. *C*, mass spectrum (MS/MS) of the predominant VDAC1 peptide associated with the 75-kDa band. *D*, immunoprecipitation of cross-linked proteins with antisera to StAR, VDAC1, VDAC2, Tom40, and StAR preimmune serum. *E*, effect of VDAC1 knockdown on pregnenolone synthesis in COS-1 cells transfected with the indicated plasmids. There was no decrease in pregnenolone with nonspecific mixtures of siRNA (*Neg*). VDAC1 knockdown did not alter pregnenolone levels in cells treated with 22(*R*)-hydroxycholesterol. *F*, effect on steroidogenesis of blocking VDAC1 with KPA in COS-1 cells transfected with the indicated vectors. *G*, measurement of accumulated pregnenolone synthesis in COS-1 cells co-transfected with StAR/F2 in the presence of KPA, atractyloside, and bongkrekic acid. *H*, transient blocking of VDAC1 stops pregnenolone synthesis in MA-10 cell mitochondria incubated with biosynthetic StAR in the presence of KPA. Mitochondria previously treated with KPA were reisolated and reincubated with unlabeled cell-free synthesized StAR, then pregnenolone synthesis was determined. Data in *panels E–H* are mean ± S.E. of three separate experiments, each performed in triplicate.

aliquoted in smaller fractions and immunoprecipitated with the indicated antibodies, as described above.

Measurement of ATP—COS-1 or MA-10 cells were serum starved for 24 h and then stimulated with 100 mM 2-deoxyglucose and 20 μM rotenone. Media were collected for radioimmunoassay, and cell lysates were analyzed for StAR and phosphoserine-StAR by Western blot. Cellular ATP content was determined using a kit according to the manufacturer's instructions (ENLITEN ATP Assay system, Promega).

RESULTS

Identification of VDAC1—StAR activity is maximal when it is affixed to the OMM (3), suggesting that StAR interacts with OMM proteins. [^{35}S]StAR synthesized in a cell-free system was chemically cross-linked to OMM proteins on freshly prepared sheep adrenal mitochondria using disuccinimidyl suberate. This consistently generated a 75-kDa consistent complex (Fig. 1*B*). Other homobifunctional cross-linkers cross-linked a band of similar size (not shown). This 75-kDa band was specific as observed on titration with different concentrations of cross-linkers (data not shown). Mass spectrometry showed that the bands at about 50 and 60 kDa did not contain StAR, and that the dark band at 50 kDa was globulin. Considering the specificity of the cross-linking products, the 75-kDa band was excised, digested with trypsin, and analyzed by mass spectrometry. In 12 experiments, 5 peptides corresponding to VDAC1 were identified (Table 1); two of these, LTFDSSFSPNTGK (Fig. 1*C*) and VTQSNFAVGYK (not shown), were seen in 10 of 12 experiments. A model structure of VDAC1 (19) indicates these two peptides are exposed to cytoplasm. Immunoprecipitation of cross-linked proteins with StAR

TABLE 1
VDAC1 peptides identified by MS/MS

Position	M_r	Sequence
53–60	833.44	VTGSLETK
256–265	1029.58	LTLSALLDGK
163–173	1212.59	**VTQSNFAVGYK**[a]
96–108	1399.68	**LTFDSSFSPNTGK**[a]
34–52	1958.82	SENGLEFTSSGSANTETTK

[a] Frequently associated with StAR.

and VDAC1 antibodies also yielded a 75-kDa band, whereas immunoprecipitation with antisera to Tom 40, PBR, or VDAC2 did not, demonstrating the specificity of the StAR-VDAC1 interaction (Fig. 1*D*). The relatively minor band above the major 75-kDa band did not yield enough protein for mass spectrometric analysis, even when multiple gel fractions were pooled together.

Role of VDAC1 in StAR Activity—To understand the significance of StAR-VDAC1 interactions, we used siRNA to knockdown VDAC1 in COS-1 cells co-transfected with expression vectors for StAR and the F2 fusion protein of the cholesterol side chain cleavage system (H_2N-P450scc-Ad-Red-Adx-COOH) (16), or for F2 and StAR lacking 62 N-terminal residues (N-62 StAR), which is as active as full-length StAR but is not imported into mitochondria (3) (Fig. 1*E*). Knockdown of VDAC1 inhibited pregnenolone synthesis, but synthesis could be restored by co-transfecting a VDAC-1 expression plasmid in which two nucleotides within the siRNA region were mutated. As an alternative to siRNA-mediated VDAC1 knockdown, cells were treated with KPA, a selective inhibitor of VDAC1 (20), reducing activity by 74% (Fig. 1*F*). VDAC1 is associated with adenine nucleotide transporter (ANT) on the IMM (21). Bongkrekic acid blocks the transfer of phosphate across the IMM and atractyloside inhibits transport of phosphate from the OMM to IMM by blocking ANT (22). Blocking of ANT with bongkrekic acid or atractyloside in COS-1 cells cotransfected with StAR and F2 showed no effect on pregnenolone synthesis (Fig. 1*G*). Water-soluble 22(*R*)-OH cholesterol, which bypasses the action of StAR, retained equivalent activity in all conditions, indicating that absence of VDAC1 did not affect P450scc. Similarly, when StAR was incubated with steroidogenic mitochondria, no pregnenolone was synthesized in the presence of KPA (Fig. 1*H*), but ANT blockers had no effect (not shown). Re-isolation of the KPA-treated mitochondria and reincubation with StAR restored pregnenolone synthesis, showing that temporary blocking of VDAC1 prevented cholesterol delivery. These results provide evidence that StAR-VDAC1 interactions are required for mitochondrial cholesterol import.

Western blotting showed that VDAC1 knockdown in StAR-transfected cells reduced expression of the 37-kDa precursor, 32-kDa intermediate, and 30-kDa mature forms of StAR, respectively, and that there is no expression of phosphorylated StAR (Fig. 2*A*). Western blotting of COS-1 cells co-transfected with F2, N-62 StAR, and a VDAC1 knockdown vector showed reduced expression of phospho-StAR, but the expression of nonphosphorylated N-62 StAR remained unaltered (Fig. 2*B*). This effect is specific to StAR, as glyceraldehyde-3-phosphate dehydrogenase and mitochondrial COX-IV remained unchanged in VDAC1 knockdown cells (Fig. 2*A*). Surprisingly, blocking VDAC1 after preincubating the cells with two different concentrations of KPA suppressed the processing of phosphorylated 32-kDa StAR to 30-kDa StAR in transfected COS-1 cells (Fig. 2*C*). Pulse-chase experiments of StAR-transfected COS-1 cells showed no processing of 32 to 30-kDa phospho-StAR in the presence of KPA, but processing of non-phosphorylated StAR was not altered (Fig. 2*D*). These results suggest that processing from 32 to 30 kDa phospho-StAR requires VDAC1.

We next addressed how VDAC1 influences StAR phosphorylation and activity. The import kinetics of [^{35}S]StAR with mitochondria from normal or VDAC-1 knockdown COS-1 cells were almost identical (Fig. 2*E*). Cell-free synthesized StAR is phosphorylated (23) and VDAC1 is necessary for the processing of StAR, thus the import of [^{35}S]StAR remained unchanged in VDAC1 knockdown cells. In the absence of VDAC1, StAR might be misfolded, rendering it susceptible to proteolytic degradation. To test this hypothesis, VDAC1 knockdown cells were incubated with MG132, lactacystin, and E64; MG132 and lactacystin are proteasomal inhibitors (24), whereas E64 is an inhibitor of cysteine proteases but not proteasomes (25). Pregnenolone synthesis was marginally increased with MG132 (Fig. 2*F*), but lactacystin had no effect on StAR activity or expression (Fig. 2*G*). Surprisingly, pregnenolone synthesis was restored to 100% with E64 (Fig. 2*F*), accompanied by restoration of 37- and 32-kDa phospho-StAR (Fig. 2*G*, *bottom panel*). Increased levels of the non-phosphorylated 37- and 32-kDa StAR were seen in cells treated with MG132 and E64 (Fig. 2*G*, *top panel*), suggesting that these proteasome inhibitors protected non-phosphorylated StAR. These data suggest that misfolded StAR cannot remain in the cytoplasm and is degraded by cysteine proteases.

Identification of PCP—It is not clear how StAR is dephosphorylated in the absence of VDAC1. Yeast mitochondria have a 32-kDa PCP that mediates phosphate transport across the membrane (26). PCP was initially identified in mitochondrial contact sites, but has also been found in the OMM (26, 27). Methotrexate binds to cytosolic dihydrofolate reductase (DHFR), so that addition of methotrexate during import of a mitochondrially targeted protein fused with DHFR at the C terminus will retain the protein at the OMM (28). To explore the potential role of PCP in StAR activity, we examined sheep adrenal mitochondrial import of [^{35}S]StAR-DHFR (1–284 StAR + 2–224 DHFR) with or without methotrexate. Analysis of digitonin lysates of the StAR-DHFR complex on a 3–16% gradient native-PAGE revealed a band of about 140 kDa containing wild-type StAR, but not the inactive mutant, R182L StAR (Fig. 3*A*). In the absence of methotrexate StAR-DHFR formed a weak complex of lower apparent molecular weight (Fig. 3*A*). The complex resisted mild proteolysis with proteinase K (Fig. 3*A*), suggesting that StAR was bound tightly. Following sucrose gradient ultracentrifugation, the complex was found in fractions 7–11 and was resistant to proteinase K, whereas fractions 12–16 were digested (Fig. 3*B*) possibly indicating that they represented aggregates. The bands in fractions 7–11 were excised, digested with trypsin, and analyzed by mass spectrometry, identifying StAR, VDAC1, and PCP (29). In 6 experiments, 7 peptides corresponding to PCP were identified (Table 2); one peptide was seen in all 6 experiments (Fig. 3*C*); no differences were seen among fractions 7–11. Co-immunoprecipitation with antisera to VDAC1, StAR, and PCP confirmed the association of PCP and VDAC1 with StAR in fractions 7–11 (Fig. 3*B*). Antisera specific for VDAC residues 151–165, StAR, or PCP supershifted [^{35}S]StAR from the complex (Fig. 3*D*),

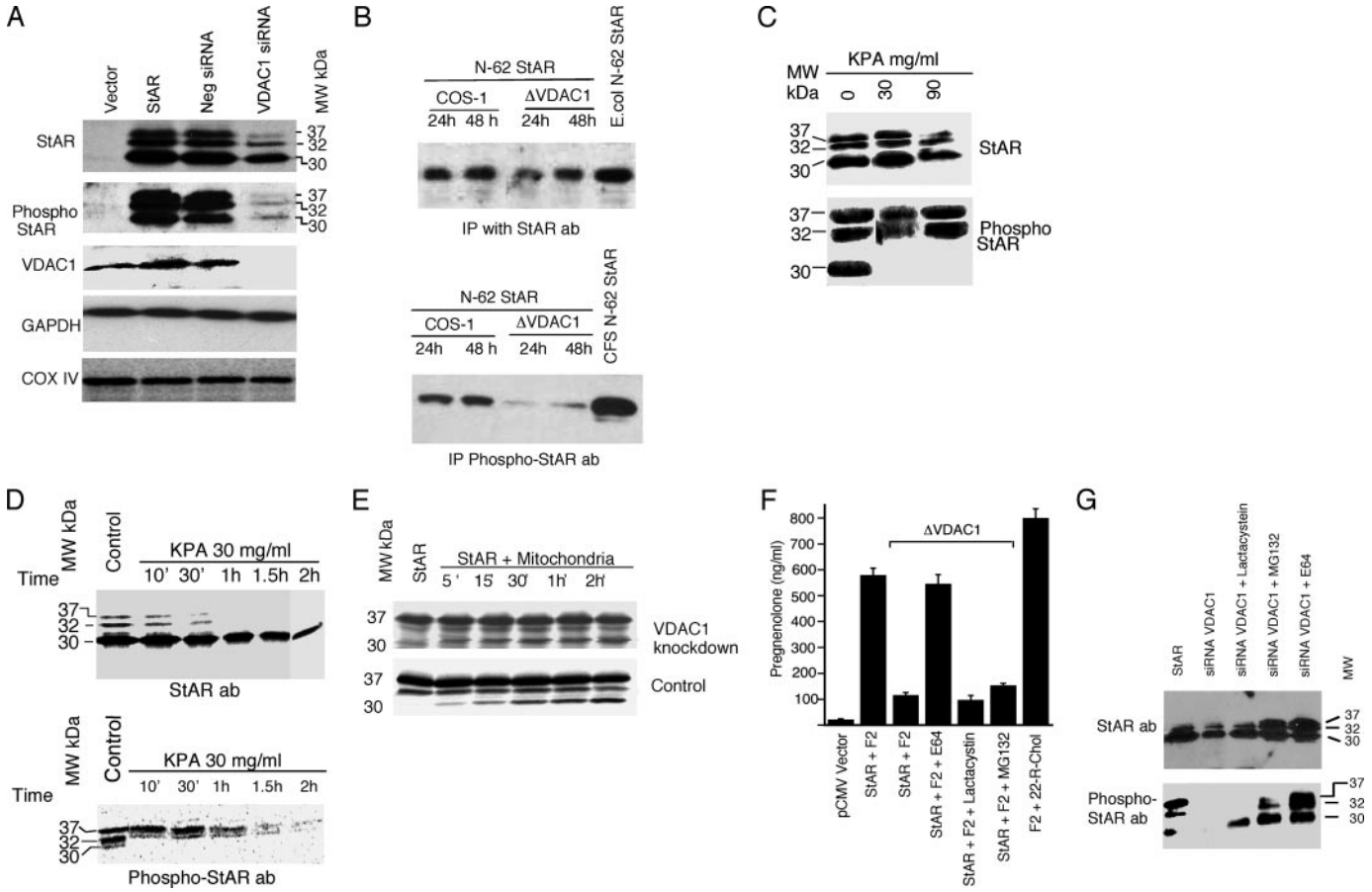

FIGURE 2. **Role of VDAC1 in pregnenolone synthesis.** *A*, Western blot of cytoplasm from VDAC1 knockdown COS-1 cells using the antisera indicated to the *left*. *B*, immunoprecipitation of StAR or phospho-StAR from VDAC1 knockdown COS-1 cells. *C*, COS-1 cells transfected with StAR were incubated with KPA, and lysates were immunoblotted with antisera to StAR and phospho-StAR. Blocking VDAC1 prevented processing of 32-kDa phospho-StAR to the 30-kDa form. *D*, pulse-chase experiments. COS-1 cells transfected with StAR were incubated in 30 mg/ml KPA, pulse labeled with [^{35}S]methionine for 45 min, then chased with cold methionine for the indicated time and immunoprecipitated with the indicated antibodies. *E*, import kinetics of StAR. [^{35}S]StAR was imported from 5 min to 2 h into COS-1 mitochondria (*bottom*) and compared with import into mitochondria from VDAC1 knockdown cells (*top*). *F*, activation of proteasomal proteases in the absence of VDAC1. Stably selected VDAC1 knockdown COS-1 cells were co-transfected with StAR and F2 and treated with the indicated protease inhibitors; StAR activity was restored by incubation with E64. *G*, immunoblotting of lysates from cells in *panel F* using antisera to StAR and phospho-StAR.

whereas preimmune serum or PBR antiserum did not, demonstrating specific association of StAR with VDAC1 and PCP. Immunoprecipitation of the digitonin extract with antibodies to StAR, VDAC1, and PCP independently confirmed the presence of these proteins in the complex (Fig. 3*E*).

Role of PCP in StAR Activity—PCP knockdown by siRNA reduced expression of PCP and phospho-StAR, partially reduced expression of the 30-kDa dephospho-StAR but did not affect VDAC1 (Fig. 4*A*). This PCP knockdown in cells cotransfected with StAR and F2 blocked pregnenolone synthesis (Fig. 4*B*), suggesting that PCP is essential for maintaining phosphorylated StAR. To determine whether PCP stimulates ATP levels, we examined the effect of ATP on StAR activity by depleting ATP via serum starvation or drug inhibition, in contrast to supplementing with exogenously added ATP. VDAC1 knockdown decreased intracellular ATP to 40% compared with control cells (Fig. 4*C*), but exogenously added ATP did not restore StAR activity (not shown). Depletion of PCP or serum starving the cells for 48 h reduced ATP levels to 10%. A similar reduction in ATP was seen after incubating cells with the ATP inhibitor 2-deoxyglucose or the electron transport inhibitor, rotenone (30) (Fig. 4*C*). As expected, incubation with rotenone or 2-deoxyglucose reduced pregnenolone synthesis similarly to knockdown of VDAC1 or PCP in COS-1 cells co-transfected with StAR and F2 (Fig. 4*D*). Consistent with the PCP knockdown experiments (Fig. 4*A*), preincubation of mitochondria with the irreversible mitochondrial phosphate carrier inhibitors, PITC or *p*-sulfo-PITC (31), blocked the import of [^{35}S]StAR (Fig. 4*E*). Similar results were obtained in response to mersalyl, a reversible sulfhydryl blocking reagent (31). The addition of mersalyl to *p*-sulfo-PITC-treated mitochondria did not rescue StAR import, but StAR import was restored by adding ATP to mitochondria preincubated with PITC (Fig. 4*E*). Import of [^{35}S]StAR into isolated mitochondria was inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (*m*CCCP), which disrupts mitochondrial membrane potential, but was not inhibited by bongkrekic acid and atractyloside (Fig. 4*F*), suggesting that ANT does not participate in StAR activity or import. These results indicate that PCP regulates phosphate availability needed for StAR phosphorylation. PCP appears to function upstream of VDAC1, as the absence of VADC1 did not completely eliminate ATP.

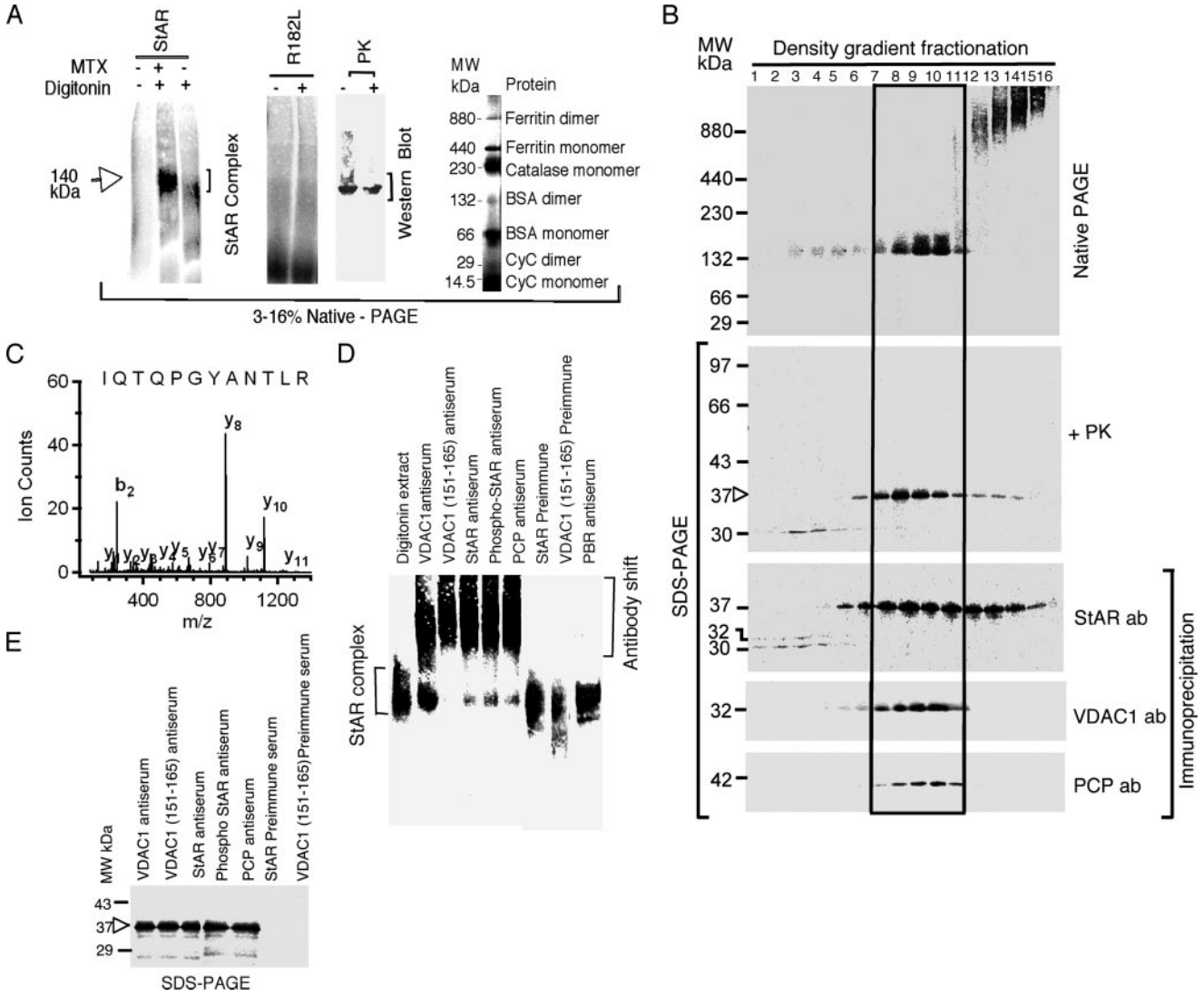

FIGURE 3. **Characterization of phosphate carrier protein associated in the StAR complex.** *A*, native gel electrophoresis of digitonin extract following import of [^{35}S]StAR into isolated mitochondria. StAR, but not the inactive StAR mutant R182L, forms a 140-kDa complex, which is resistant to mild proteolysis with proteinase K (*PK*). Size markers are shown to the *right*. *B*, sucrose density gradient analysis of the 140-kDa complex. Size markers are shown to the *left* in all panels. *Top panel*, autoradiogram of gradient native-PAGE gel of sucrose gradient fractions. *Second panel*, SDS-PAGE gel of fractions from the same amount of protein displayed in the *top panel* subjected to mild proteolysis with PK. The purified complex from *lanes 7* to *11* is resistant to proteolysis. *Third* to *fifth panels*, immunoprecipitation of the sucrose density gradient fractions with the indicated antibodies followed by SDS-PAGE. *C*, mass spectrometric identification of the predominant peptide, showing it is from PCP. *D*, antibody shifts of the digitonin extract of the 140-kDa complex. The digitonin-extracted complex was incubated with the indicated antibodies and analyzed by native gradient gel electrophoresis. *E*, immunoprecipitation. The digitonin-extracted 140-kDa complex was immunoprecipitated with the indicated antibodies and separated by SDS-PAGE.

TABLE 2

PCP peptides identified by MS/MS

The sequences obtained from sheep adrenal mitochondria were compared to the bovine sequence (Swiss Prot Accession number Q7Z7N7 (same as gb/AAH00998 and others)) as there is no sheep sequence in the database. The bold letters indicate unmatched residues, which may represent bovine-sheep differences.

Position	Mol WT	Sequence
107–113	860.39	M_{ox}QVDPQK
310–318	875.47	GSSAS**N**VLK
221–228	1012.49	M_{ox}YKEEGLK
152–159	1035.48	FGFYEVFK
204–215	1360.65	IQTQPGYANTLR
356–369	1602.82	LPRPPPPEM$_{ox}$PESLK
66–77	1491.59	AVEEYSCE**Y**GSAK

DISCUSSION

Despite extensive study, the mechanism of StAR action remains unclear. StAR acts exclusively on the OMM prior to import (3), must undergo a structural transition to exert activity (9, 32), and phosphorylation of Ser^{195} augments its steroidogenic activity (4). The ability of StAR to transfer cholesterol between membranes is distinct from its ability to induce steroidogenesis (33). The proposal that StAR acts through an OMM receptor was challenged by data showing it had activity with mitochondria subjected to proteolysis (34). Nevertheless, substantial data suggest that cholesterol import into mitochondria requires the 18-kDa PBR on the OMM. PBR has five transmembrane domains; its N terminus binds benzodiazepines that stimulate steroidogenesis and its C terminus binds cholesterol (7). A functional association with StAR is indicated by the loss of StAR activity in PBR-knockdown cells (8). Several proteins appear to interact with PBR, including VDAC1 and ANT (21), a 10-kDa protein (35), PRAX-1 (36), and PBR-

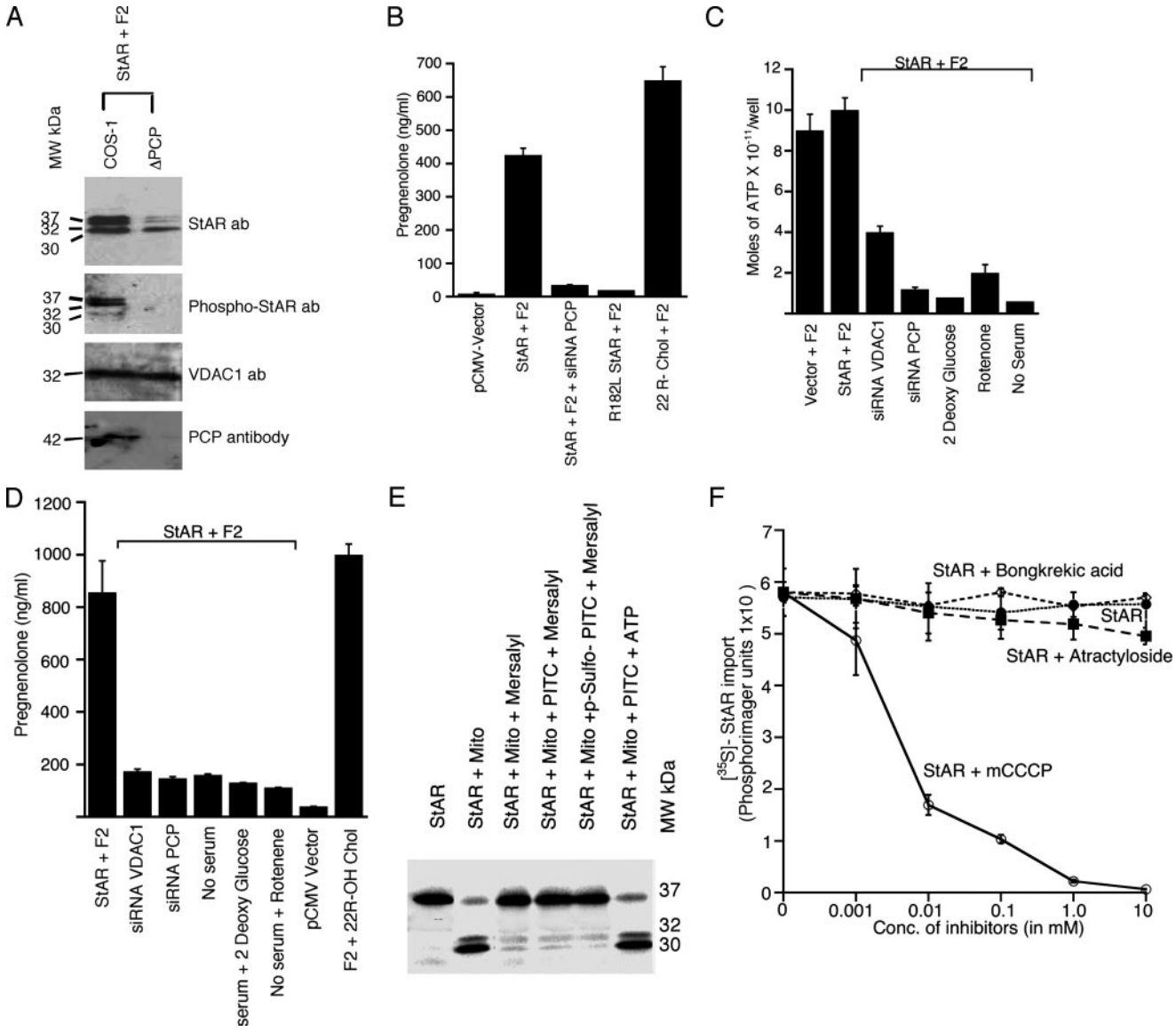

FIGURE 4. **Role of phosphate carrier protein in pregnenolone synthesis.** *A*, Western blot. Lysates collected from cells in *panel B* were immunoblotted with the indicated antibodies; Δ*PCP* denotes PCP knockdown by siRNA. *B*, pregnenolone synthesis by PCP knockdown COS-1 cells co-transfected with StAR and F2. Incubation with 22(*R*)-hydroxycholesterol served as a positive control. *C*, measurement of ATP in untreated VDAC1 knockdown or PCP knockdown COS-1 cells expressing StAR and F2, and in serum-starved COS-1 cells treated with 2-deoxyglucose or rotenone. *D*, measurement of pregnenolone synthesis in COS-1 cells transfected with StAR and F2 that were serum starved, then treated with 2-deoxyglucose or rotenone. PCP and VDAC1 knockdown cells served as negative controls, and F2-transfected cells treated with 22(*R*)-hydroxycholesterol served as a positive control. *E*, import of [^{35}S]StAR after blocking ATP transport with mersalyl, PITC, or *p*-sulfo-PITC. The import of StAR was interrupted by blocking PCP with PITC; the addition of exogenous ATP restored StAR import in the presence of PITC. *F*, [^{35}S]StAR was imported into isolated mitochondria in the presence and absence of mCCCP, bongkrekic acid, or atractylosidase. The intensity of the imported 30-kDa band was measured by phosphorimager and plotted against the concentrations of the drugs. Data in *A*, *C*, *D*, and *F* are expressed as mean ± S.E. of three separate experiments, each performed in triplicate.

associated protein 7 (PAP7) (37). Photoaffinity labeled PBR forms a complex of 170–210 kDa (38) or 200–240 kDa (39), but these complexes did not include StAR. Therefore the nature of the StAR receptor has been unclear. We have now shown that StAR directly interacts with VDAC1 and PCP on the OMM.

These observations are wholly consistent with the literature implicating PBR in the action of StAR to induce steroidogenesis. The association with PCP is consistent with the need to phosphorylate StAR to achieve maximal activity. StAR appears to be phosphorylated by PKA, and PAP7 appears to act as an anchoring protein to promote the interaction of PBR and PKA regulatory subunit 1a (PKAR1α) (37). PCP may play a role in delivering phosphate to PKAR1a (27). Only the cytoplasmically exposed domains of VDAC1 interacted with StAR, and the C terminus of StAR formed contacts with VDAC1, consistent with data that this domain of StAR also interacts with synthetic membrane models of the OMM (40). We suggest that StAR acts by interacting with VDAC1, which is part of a larger OMM complex including StAR, VDAC1, PCP, PBR, PAP7, PKAR1α, and possibly other proteins. It remains unclear whether VDAC1 forms the channel through which cholesterol passes or if this function is served by PBR. As VDAC1 knock-out mice are viable (41) but PBR knockouts are embryonic lethal (7), we sug-

gest that the channel is formed by PBR in association with other proteins.

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