Protein-Protein Interactions Mediate Mitochondrial Cholesterol Transport and Steroid Biosynthesis*

Received for publication, September 12, 2006, and in revised form, October 16, 2006 Published, JBC Papers in Press, October 18, 2006, DOI 10.1074/jbc.M608820200

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Transport of cholesterol into the mitochondria is the rate-determining, hormone-sensitive step in steroid biosynthesis. Here we report that the mechanism underlying mitochondrial cholesterol transport involves the formation of a macromolecular signaling complex composed of the outer mitochondrial membrane translocator protein (TSPO), previously known as peripheral-type benzodiazepine receptor; the TSPO-associated protein PAP7, which binds and brings to mitochondria the regulatory subunit R1α (PKA) of the cAMP-dependent protein kinase (PKAR1α); and the hormone-induced PKA substrate, steroidogenic acute regulatory protein (Star). Hormone treatment of MA-10 Leydig cells induced the co-localization of TSPO, PAP7, PKAR1α, and Star in mitochondria, visualized by confocal microscopy, and the formation in living cells of a high molecular weight multimeric complex identified using photoactivable amino acids. The hormone-induced recruitment of exogenous TSPO in this complex was found to parallel the increased presence of 7-azido-5α-cholestan-3β-ol in the samples. Co-expression of Tspo, PAP7, PKAR1α, and Star genes resulted in the stimulation of steroid formation in both steroidogenic MA-10 and non-steroidogenic COS-F2-130 cells that were engineered to metabolize cholesterol. Disruption of these protein-protein interactions and specifically the PKAR1α-PAP7 and PAP7-TSPO interactions, using PAP7 mutants where the N0 area homologous to dual A-kinase-anchoring protein-1 or the acyl-CoA signature motif were deleted or using the peptide Ht31 known to disrupt the anchoring of PKA, inhibited both basal and hormone-induced steroidogenesis. These results suggest that the initiation of cAMP-induced protein-protein interactions results in the formation of a multivalent scaffold in the outer mitochondrial membrane that mediates the effect of hormones on mitochondrial cholesterol transport and steroidogenesis.

Steroids are formed by several successive enzymatic transformations of the substrate cholesterol in the mitochondria and endoplasmic reticulum (1). The rate-determining step for steroidogenesis is the transport of the precursor, cholesterol, from intracellular sources into the mitochondria (2, 3). This step is regulated by trophic hormones, such as adrenocorticotropic hormone, in adrenocortical cells and luteinizing hormone in testicular Leydig and ovarian cells that act via G-protein-coupled receptors to activate adenylate cyclase and induce the formation of cAMP (1–3). Four molecules of cAMP bind to the regulatory (R)2 subunits of the cAMP-dependent protein kinase (PKA) holoenzyme to release the catalytic subunits that phosphorylate specific substrates/effectors, thus transducing and amplifying the signal transmitted by the hormone (2–4). Protein phosphorylation has been shown to be one of the regulatory steps in hormone-stimulated steroid formation (2, 5). This is a highly efficient and rapid process where minimal amounts of cAMP are needed to induce a maximal rate of cholesterol transport and steroid synthesis within minutes upon exposure of the cells to hormones (6). The mechanism underlying this amplification process targeted to the mitochondria remains unknown. However, a number of proteins have been shown to be critical in this process.

The steroidogenic acute regulatory protein (Star) is a hormone-sensitive, 37-kDa protein that contains an N-terminal mitochondrial signal sequence (3, 7). In both gonadal and adrenal cells, de novo synthesis of Star parallels maximal steroid synthesis in response to trophic hormones (3, 7). The newly synthesized Star is functional and exhibits cholesterol transfer activity (8) that acts outside of the mitochondria to stimulate steroidogenesis (9, 10). This cytosolic preprotein is cleaved in mitochondrial membrane contact sites to produce the 30-kDa “mature” Star protein, an event that terminates its action (10). Thus, Star may function by activating either a mitochondrial receptor or transport mechanism; mitochondrial import and proteolysis may terminate its action. Such cAMP-induced phosphorylation of Star has also been shown to be important for its function (11).

The translocator protein (18 kDa; TSPO), previously known as the peripheral-type benzodiazepine receptor (12), is a high affinity cholesterol- and drug-binding protein that is abundant in steroidogenic cells, localized to the outer mitochondrial membrane, and enriched in the outer/inner membrane contact

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8 This work was supported by NICHD, National Institutes of Health Grant HD37031. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE 1
Primers for cDNA cloning and mammalian expression plasmid construction

| Gene  | Accession no. | Primers* | Vector inserted       |
|-------|---------------|----------|-----------------------|
| mPAP7 | AF022770      | mPAP7-F2, 5'-AAAGTCGACATGTCGCGGCGGCGCTGT | pCR4-TOPO             |
|       |               | mPAP7-R2, 5'-CGAGAGGCGCCAAACAAAGTCA       |                       |
|       |               | mPAP7-F1, 5'-CATGAGTATCGATGTCGCGGCGGCGCTTT |                       |
|       |               | mPAP7-R1, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT |                       |
|       |               | mTSPO-F2, 5'-CGAGAGGCGCCAAACAAAGTCA       | pEGFP-C3, pEYFP-C1, PEYFP-N1, pCDNA3.1(−) |
|       |               | mTSPO-R2, 5'-CATGAGTATCGATGTCGCGGCGGCGCTTT | pCR4-TOPO             |
| mTSPO | D21207        | mTSPO-F1, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT |                       |
|       |               | mTSPO-R1, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT | pEGFP-C3, pCDNA3.1(+)  |
| mPKARlα| XM_126543   | mPKARIA-F1, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT | pCR4-TOPO             |
|       |               | mPKARIA-R1, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT |                       |
|       |               | mPKARIA-F3, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT |                       |
|       |               | mPKARIA-R3, 5'-GCGCCACAGCTGCAGGACTCAGGACCTT |                       |
| mStAR | NM_011485    | mStAR-F1, 5'-GGGTGTGGGA to AGGTTCAGGGGGAGGTGTGGGA | pCR4-TOPO             |
|       |               | mStAR-R1, 5'-GGGTGTGGGA to AGGTTCAGGGGGAGGTGTGGGA |                       |
|       |               | mStAR-F2, 5'-GGGTGTGGGA to AGGTTCAGGGGGAGGTGTGGGA |                       |
|       |               | mStAR-R2, 5'-GGGTGTGGGA to AGGTTCAGGGGGAGGTGTGGGA |                       |

* Restriction sites in primer adaptor are underlined.

sites (12, 13). Ligand binding to TSPO results in the stimulation of mitochondrial pregnenolone formation (13). Hormones together with cAMP induce a functional and topographical reorganization (polymerization) of TSPO (14, 15). It was recently shown that the action of STAR on the outer mitochondrial membrane is mediated by TSPO (16), suggesting that there may be a cAMP-regulated STAR-TSPO interaction at the mitochondrial level.

In search of cytosolic proteins that interact with and regulate TSPO, we identified a TSPO-associated protein, PAP7, that is found primarily in Golgi associated with mitochondria and binds the regulatory subunit Rlα of PKA (17, 18). It has been suggested that PKARlα is compartmentalized in Leydig cells so that it has preferential access to endogenously produced cAMP (19). The compartmentalization of PKA, mediated through the specific binding of R subunits to various organelles, has been proposed as a mechanism to target the response to cAMP (20).

In this regard, PAP7 is a PKARIα-protein (A-kinaseanchoring protein (AKAP)) that targets the kinase to TSPO-rich mitochondria. Recently the AKAP family was described to anchor PKA to various organelles, has been proposed as a mechanism to target the response to cAMP (20). In this regard, PAP7 is a PKARIα-anchoring protein (A-kinase-anchoring protein (AKAP)) that targets the kinase to TSPO-rich mitochondria. Recently the AKAP family was described to anchor PKA to various organelles, has been proposed as a mechanism to target the response to cAMP (20).

Reduction or deletion of the Star, Tspos, or Pkap genes products in either in vivo or in vitro models resulted in the arrest of both basal and hormone-induced steroidogenesis (12, 16, 17, 24–26), demonstrating the respective importance of these proteins in steroidogenesis. Here we show that hormonal stimulation leads to the formation of a StAR-PKARIα-PAP7-TSPO macromolecular signaling complex in the outer mitochondrial membrane that mediates and amplifies the cAMP signal that initiates cholesterol transport and steroid formation.

EXPERIMENTAL PROCEDURES

Cell Culture—MA-10 cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 5% heat-inactivated fetal calf serum and 2.5% horse serum (16). COS-F2-130 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (27).

cDNA Cloning and Plasmid Construction—To clone cDNA sequences of the mouse PAP7, Tspos, PKARlα, and Star genes, which include the complete coding regions, primers were designed and synthesized based on the sequences deposited in GenBank™ (Table 1). Total RNA from MA-10 cells was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed. Sequences were amplified with the Advantage 2 PCR polymerase (BD Biosciences). Optimal cycling parameters for the first round of PCR were 95 °C for 30 s, 72 °C for 3 min for five cycles, 95 °C for 30 s, 70 °C for 3 min for five cycles, 95 °C for 30 s, 68 °C for 3 min for 25 cycles, and a 10-min extension at 72 °C. PCR products were inserted into the TA-TOPO cloning vector (Invitrogen) and sequenced using M13-forward and M13-reverse primers. The ABI PRISM dye terminator cycle sequencing ready reaction kit and sequencer (Applied Biosystems, Foster City, CA) were used for nucleotide sequencing at the Sequencing Core Facility (Georgetown University). Constructs for mammalian expression either alone or fused with green fluorescent protein (GFP), yellow fluorescent protein (YFP), or cyan fluorescent protein (CFP) (purchased from BD Biosciences) were used; the primers for nested PCR were linked with certain restriction nucleotide cleavage sites (Table 1). The parameters were identical to the first round. PCR products were purified using the GeneSpin kit (Qbiogene, Irvine, CA), digested by the relevant enzymes, and subjected to sequencing reactions for further confirmation.

Construction of PAP7 Mutants—Two deletions, which were called N0 homologous deletion and acyl-CoA-binding motif deletion, were designed based on the peptide sequence of mouse PAP7 (17, 18). To create the N0 homologous deletion, two fragments that connect with the N0 homologous area at both the N- and C-terminal ends were amplified using two primer sets: 5′-ATCATGGGCGAACAAGCAGAAGACG and 5′-GCTCTAGCCGGCGGCTT and 5′-GATGCGGAAAGCCTTCTACATTGATG and 5′-AGGTTCAGGGGGAGGTTGAGGA. After purification, the two fragments were ligated using a Rapid Ligation kit (Roche Applied Science). The ligation product was purified and subjected to PCR as the template using primers 5′-ATCATGGGCGAACAAGCAGAAGACG and 5′-AGGTTCAGGGGGAGGTTGAGGA to amplify the coding region without the N0 homologous region.
Following another purification step, the amplified N0 homolo-
gous deletion fragment was digested by EcoRI and BamHI and
inserted into mammalian expressing vector pcDNA3.1(-). The acyl-CoA deletion was created using a similar methodology.
The primers to amplify the N- and C-terminal fragments were
5'-ATCATGGCCGACAAGCAGAAGACG and 5'-GCCG-
TAAAGCCTCTCCAGGCGCAAG and 5'-GCATATGTT-
GCGTCCCCACAGAATAGA and 5'-AGGTTCCAGGGGAG-
GTGTGGGA. The primers for the final round of PCR were the
same as those of the N0 homologous deletion. Advantage 2 PCR
polymerase (BD Biosciences) was used for sequence amplifica-
tion, and the cycling parameters were 95 °C for 30 s, 61 °C for
15 s, 72 °C for 1 min for five cycles, 95 °C for 30 s, 58 °C for 15 s,
72 °C for 1 min for 30 cycles, and a 10-min extension at 72 °C.

Transient Transfection—COS-F2-130 and MA-10 cells were
transfected using the FuGENE 6 transfection reagent (Roche
Applied Science) and Lipofectamine 2000 (Invitrogen), respec-
tively. Cells were plated 1 day before transfection. The con-
struction of fusion proteins with PAP7, TSPO, and PKARI
were used. For the co-localization of PAP7 or TSPO
Golgi apparatus protein syntaxin-6 (1:150 dilution; BD Bio-
sciences) were used. For the co-localization of PAP7 or TSPO
Golgi apparatus, mouse PAP7 rabbit polyclonal antibody (1:150
dilution; Ref. 17) and a mouse monoclonal antibody against the

Confocal Microscopy—For immunocytochemical studies,
MA-10 cells were cultured on coverslips in 12-well plates. The
next day, cells were either treated with the indicated hormone
or compound or subjected to staining. Following washing with
PBS, slides were either stained with MitoTracker® Red
CMXRos (working concentration, 100 nM; Molecular Probes,
Inc., Eugene, OR) or fixed with 3.7% formaldehyde for 10 min.
Slides were incubated for 1 h at room temperature with various
primary antibodies. For the co-localization of PAP7 with the
Golgi apparatus, mouse PAP7 rabbit polyclonal antibody (1:150
dilution; Ref. 17) and a mouse monoclonal antibody against the
Golgi apparatus protein syntaxin-6 (1:150 dilution; BD Bio-
sciences) were used. For the co-localization of PAP7 or TSPO
with the mitochondria, the slides were incubated with anti-
PAP7 or rabbit polyclonal anti-TSPO (1:150 dilution; Ref. 28) as
primary antibody, respectively. The monoclonal antibody anti-
PKARIα (1:100 dilution) (BD Biosciences) was used to study the
co-localization of PKARIα with either PAP7 or TSPO. Slides
were then rinsed in PBS three times and incubated for 1 h with
two secondary antibodies, fluorescein isothiocyanate–goat anti-
rabbit IgG (1:200 dilution; Zymed Laboratories Inc., South San
Francisco, CA) and rhodamine Red-X-conjugated AffiniPure
goat anti-mouse IgG (1:200 dilution; Jackson Immunoresearch
Laboratories, West Grove, PA). Antibodies were diluted in PBS
containing 0.1% gelatin, 0.1% Triton X-100, and 1% normal goat
serum. Slides were then mounted with the Prolong Antifade kit
(Molecular Probes, Inc.) and observed under an Olympus Flu-
view laser scanning microscope.

Steroid Biosynthesis—MA-10 cells were plated onto 96-well
plates at 2.5 × 10⁴ cells/well. Media were replaced with serum-
free media after 24 h, and cells were treated with or without 10
µg/ml brefeldin A (BFA) (Sigma) for 30 min. Cells were washed
with serum-free media and treated with media with or without
the indicated concentrations of human chorionic gonadotropin
(hCG) for the indicated time periods. Purified hCG was a gift
from Dr. A. F. Parlow (National Hormone and Pituitary Pro-
gram, National Institutes of Health). At the end of the incuba-
tion, media were collected, and progesterone levels were deter-
mined by radioimmunoassay (RIA) as described previously (16,
17). Anti-progesterone antisera were from ICN Pharmaceuti-
cals (Costa Mesa, CA) and [1,2,6,7-³H]progesterone (specific
activity, 94.1 Ci/mmol) was from PerkinElmer Life Sciences.
Progesterone production was normalized for the amount of
protein in each well as determined by the method of Bradford
(29). RIA data were analyzed using the MultiCalc software
(EGG & Wallac, Inc., Gaithersburg, MD).

To determine steroidogenesis in cells transfected with the
various constructs, COS-F2-130 and MA-10 cells were plated
onto 48-well plates at 5 × 10⁴ cells/well. Culture media were
collected after 48 h of transient transfection. Cells were washed
and incubated with the indicated concentrations of hCG in
serum-free media for the indicated time periods. In some
experiments, the hydrosoluble analog of cholesterol, (22R)-hy-
droxysterol, was used to assess maximal steroidogenic capa-
city of the cells independent of hormonal stimuli (data not
shown). Pregnenolone production in COS-F2-130 cells and
pregnenolone and progesterone production in MA-10 cells
were measured by RIA. Anti-pregnenolone antisera were from
ICN Pharmaceuticals and [7-³H]pregnenolone (specific activ-
ity, 17.5 Ci/mmol) was from PerkinElmer Life Sciences.

To determine the optimal concentration of stearated pep-
tides, Ht31 and Ht31P (Promega, Madison, WI), in MA-10
cells, increasing concentrations (1–50 µM) of the peptides were
added to the culture medium for 2 h in the presence and
absence of saturating concentrations of hCG. Progesterone
produced was measured by RIA. Based on the results obtained
with the bioactive peptide Ht31, a concentration of 25 µM
was chosen and used to test cells in the presence of increasing
concentrations of hCG for 2 h. In separate experiments, Ht31
and Ht31P were used to test MA-10 cells 24 h post-transfec-
tion with the various constructs under investigation in the pre-
sence or absence of hCG for 2 h.

Immunoprecipitation—MA-10 cells were plated in 6-well
plates 1 day before transfection. Each well was transfected with
4 µg of pEGFP-Tspo, pEGFP-Pkarla, or empty vector. Forty-
eight hours later, cells were washed and treated with or without
saturating concentrations (50 ng/ml) of hCG for 30 min. Each
treatment was performed in three parallel wells. At the end of
the treatment, cells were then incubated for 30 min at 4 °C with
lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM
NaCl) supplemented with a protease inhibitor mixture (Sigma).

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Samples were precleared by incubation with protein G-Sepharose (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for 1 h at 4 °C, centrifuged for 30 s at 14,000 rpm, and treated with anti-GFP antibody (Sigma) overnight at 4 °C. The samples were then incubated with protein G-Sepharose for 2 h at 4 °C. Precipitates were washed three times with lysis buffer, resuspended in non-reduced sample buffer, and centrifuged for 30 s at 14,000 rpm. Samples were separated on a 4–20% Tris-glycine polyacrylamide gel under native conditions, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and blotted with anti-STAR (1:1000; Ref. 16) antiserum. Immunoreactive proteins were visualized using an ECL kit (Amersham Biosciences). Image densitometric analysis of the StAR-immunoreactive protein band was performed using the OptiQuant image analysis software (PerkinElmer Life Sciences).

Photolabeling—MA-10 cells were plated onto 6-well plates. Twenty-four hours later, the media were replaced with media deficient in both leucine and methionine (BIOSOURCE, Camarillo, CA) supplemented with 10% fetal calf serum. Photoleucine and photomethionine, donated by Dr. Thiele (Max Plank Institute, Dresden, Germany), were added to the media at a concentration of 4 mM and incubated for 24 h (30). At the end of the incubation, cells were washed and treated with media containing 50 ng/ml hCG for the indicated periods of time. Cells were washed with PBS and irradiated with UV light using a 100-watt mercury lamp (Olympus) for 15 min in PBS containing 50 ng/ml hCG; a filter was used to remove wavelengths of light below 350 nm, allowing a peak at 365 nm. Cells were then harvested, and proteins were separated on a 4–20% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with either anti-TSPO (1:10,000; Trevigen Inc., Gaithersburg, MD), anti-glyceraldehyde-3-phosphate dehydrogenase (1:10,000), anti-PAP7 (1:1500), anti-PKARI (1:500; Abcam Inc.), or anti-glyceraldehyde-3-phosphate dehydrogenase (1:1000), anti-PAP7 (1:1500), anti-PKARI (1:500; Abcam Inc.), or anti-glyceraldehyde-3-phosphate dehydrogenase (1:10,000; Trevigen Inc., Gaithersburg, MD). Visualization and image analysis of the immunoreactive proteins were performed as described above.

In parallel experiments, COS-F2-130 cells were transfected with Star cDNA, and 24 h later, media were replaced with media deficient in leucine and methionine supplemented with 10% fetal calf serum. Photoleucine and photomethionine (4 mM) were added to the media and incubated for 24 h. Cells were washed with PBS and irradiated with UV light using a 100-watt mercury lamp (Olympus) for 15 min in PBS containing 50 ng/ml hCG; a filter was used to remove wavelengths of light below 350 nm, allowing a peak at 365 nm. Cells were then harvested, and proteins were separated on a 4–20% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with anti-TSPO (1:10,000), anti-PAP7 (1:1500), anti-PKARI (1:250; BD Biosciences), anti-STAR (1:200), anti-VDAC1 (1:1000; Abcam Inc., Cambridge, MA), anti-adenine nucleotide transporter (ANT) (1:500; Abcam Inc.), or anti-glyceraldehyde-3-phosphate dehydrogenase (1:10,000; Trevigen Inc., Gaithersburg, MD). Visualization and image analysis of the immunoreactive proteins were performed as described above.

To investigate the role of cholesterol in the formation of the 240-kDa complex, the modified cholesterol compound, 7-azido-5α-[3,5,6-3H]cholestan-3β-ol ([3H]photocholesterol, from American Radiolabeled Chemicals Inc. (St. Louis, MO), was used. MA-10 cells were transfected with pEGFP-Tspo, and 24 h later, 5 μCi (83.5 pmol) of [3H]photocholesterol were added to the media together with the artificial amino acids and incubated for 24 h. Cells were treated with hCG and irradiated with UV light as described above. At the end of the treatment, cells were harvested and immunoprecipitated with anti-GFP antibodies. After separation of the precipitates on an SDS-polyacrylamide gel and transfer onto a polyvinylidene difluoride membrane, the membrane was blotted with anti-TSPO. Radioactivity in protein precipitates prior to electrophoresis was measured by liquid scintillation spectroscopy.

Statistical Analysis—Statistical analysis was performed using the unpaired t test and analysis of variance with the Instat (version 3.0) package from GraphPad, Inc. (San Diego, CA).

RESULTS

Subcellular Localization of PAP7, TSPO, PKAR1α, and StAR: Effect of hCG Treatment—To identify the subcellular localization of PAP7, TSPO, and PKAR1α in control cells, the cDNAs of these three genes were ligated into pEGFP vectors, and the constructs were transfected into both MA-10 and COS-F2-130 cells. MA-10 mouse tumor Leydig cells are a widely used cell model to study the hormonal induction of steroidogenesis. COS1-F2-130 monkey kidney cells are derived from COS-1 cells that stably express the human cholesterol side chain cleavage system F2, including cytochrome P450 side chain cleavage (P450scC; CYP11A1), the first enzyme in steroid biosynthesis that is located in the inner mitochondrial membrane, together with the electron transport proteins adrenodoxin and adrenodoxin reductase and thus provide a non-steroidogenic system in which cholesterol is converted into pregnenolone (27). PAP7, TSPO, and PKAR1α showed similar expression patterns in MA-10 and COS-F2-130 cells. Under basal conditions, PKAR1α was mainly found in the cytosol, whereas endogenous TSPO was localized to the mitochondria (Fig. 1D). In agreement with previous findings (18), the expression of transfected PAP7 was targeted to the Golgi apparatus (Fig. 1A). These findings were further confirmed in co-localization studies of endogenous PAP7 and either MitoTracker Red CMXRs (MitoTracker), a mitochondrial marker, or syntaxin-6, a Golgi apparatus marker (Fig. 1B). Both living color display and immunofluorescence confocal microscopy demonstrated that the main subcellular locations of PAP7 are the trans-Golgi apparatus and the mitochondria, respectively, although its presence in the endoplasmic reticulum cannot be excluded (Fig. 1, A and B).

The mitochondrial localization of PAP7 is in agreement with our data showing its association with TSPO (17).

As noted above, PAP7 was identified as a TSPO-associated protein that also binds PKAR1α (17). Triggering the cAMP-PKA pathway may affect the compartmentalization of PAP7. It is now well established that the effect of hCG on Leydig cell steroidogenesis is mediated by cAMP. Treatment of MA-10 cells with saturating concentrations (50 ng/ml) of hCG resulted in the redistribution of PAP7 from the Golgi to the mitochondria as seen by its reduced co-localization with syntaxin-6 and its increased co-localization with MitoTracker (Fig. 1B). This hCG-induced redistribution of PAP7 was seen as early as 10 min after the addition of hormone and increased with exposure time, reaching maximal levels at 2 h.

To further validate these data, we investigated the effects of BFA on the subcellular distribution of PAP7 in MA-10 cells. BFA is a fungal metabolite that disrupts both the structure and function of the Golgi apparatus (31, 32). Fig. 1C (left) illustrates the effects of a 30-min pretreatment with 10 μg/ml BFA followed by a treatment with hCG on the distribution of both
FIGURE 1. Subcellular localization of PAP7, TSPO, and PKAR1α: effects of hCG and brefeldin. A, subcellular localization of mouse PAP7, PKAR1α, and TSPO expressed as GFP fusion proteins in MA-10 and COS-F2-130 cells. B, time course effects of hCG treatment on PAP7 subcellular localization. MA-10 cells were treated with or without 50 ng/ml hCG for 10, 30, and 120 min. At the end of the incubation, cells were double stained for PAP7 and syntaxin-6 or MitoTracker. C, effects of BFA on the hCG-induced changes in the subcellular localization of PAP7. MA-10 cells were incubated in culture medium with 10 μg/ml BFA for 30 min, and then the medium was changed to fresh medium containing vehicle or 50 ng/ml hCG for 10, 30, and 120 min. Cells were double stained for PAP7 and syntaxin-6 or MitoTracker. D, effects of BFA on the subcellular localization of TSPO. Confocal microscopy images show the co-localization of TSPO and MitoTracker (top panels). The middle panels show the effect of 30 min of BFA (10 μg/ml) treatment on MA-10 cells. The cells shown in the bottom panels were treated with BFA (10 μg/ml) for 30 min followed by hCG (50 ng/ml) for 120 min. All cells were double stained for TSPO and MitoTracker. E, effects of BFA on hCG-stimulated steroidogenesis. MA-10 cells were incubated with 10 μg/ml BFA for 30 min followed by treatment with hCG (50 ng/ml) for the indicated time points. Media were collected, and progesterone levels were determined by RIA. Results are means ± S.D. from four independent experiments, and each was conducted in triplicate. The effects of BFA were statistically significant (p < 0.05 at 30 min and p < 0.001 at both 90 and 120 min). Scale bars represent 30 μm (A) and 20 μm (B, C, and D).
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PAP7 and syntaxin-6. In control cells, PAP7 was mainly associated with the region of the Golgi apparatus where syntaxin-6 was co-localized (Fig. 1B). BFA treatment induced the redistribution of PAP7 into both the cytosol (Fig. 1C) and the mitochondria as shown by its increased co-localization with MitoTracker (Fig. 1C, right, and compare with Fig. 1B, 0 min). Withdrawal of BFA followed by treatment with hCG resulted in the increased translocation of PAP7 to the mitochondria (Fig. 1C, right). The response to hCG treatment was rapid, and the translocation of PAP7 to the mitochondria was maximal after 10 min (Fig. 1C). The disrupting effect of BFA could be reversed over time, and the Golgi apparatus was reassembled 2 h after drug withdrawal (Fig. 1C) in agreement with other reports (31, 33). However, due to the presence of hCG 120 min after withdrawal of BFA, most of the PAP7 was still in the cytoplasm, showing higher co-localization with the mitochondria compared with control cells (Fig. 1C). As expected, after a 2-h treatment with hCG, both mitochondria and PAP7 were located around the nuclei (Fig. 1B). These data indicated that 2 h after withdrawal of BFA, although the Golgi apparatus was reassembled, PAP7 remained associated with the mitochondria due to the strong activation signal from the hCG treatment. BFA treatment did not affect TSPO distribution in the mitochondria (Fig. 1D). In control MA-10 cells, TSPO was mainly found in the mitochondria (Fig. 1D). BFA treatment and subsequent stimulation of the cells with hCG for 30 min did not affect TSPO localization in the mitochondria (Fig. 1D).

To detect the effects of BFA on steroidogenesis, MA-10 cells were treated with or without 10 μg/ml BFA for 30 min followed by hCG stimulation (Fig. 1E). Progesterone that accumulated in the media was measured by RIA. BFA increased the hCG-induced steroid formation by MA-10 cells. Because PAP7 is translocated in a hormone-dependent manner from the Golgi apparatus to the mitochondria where it binds to TSPO and we previously showed that it binds PKAR1α (17), we investigated the spatial relationship between TSPO and PKAR1α in the mitochondria. For this experiment, we generated cDNA expression vectors for CFP and YFP with C- or N-terminal fusion proteins of PAP7 and PKAR1α and a CFP N-terminal fusion protein of TSPO for expression in MA-10 cells (Fig. 2A). Co-transfection of YFP C- or N-terminal PAP7 fusion proteins with a CFP C-terminal TSPO fusion protein in living cells resulted in partial co-localization of the proteins (Fig. 2A, c and f). Expression of a YFP N-terminal PKAR1α fusion protein with a CFP N-terminal TSPO fusion protein also showed partial co-localization to the mitochondria (Fig. 2Ai). The same result was also observed for the co-localization of TSPO with PKAR1α by immunocytochemistry (Fig. 2Bi). Treatment of cells with 50 ng/ml hCG for 2 h resulted in the increased co-localization of PKAR1α with TSPO (Fig. 2Bii). We further investigated the spatial relationship between PAP7 and PKAR1α and found that they also co-localized (Fig. 2Cr); this event was increased by hCG treatment (Fig. 2Cu).

Treatment of MA-10 cells with hCG results in the rapid induction and targeting of StAR to the mitochondria where it exerts its activity (3, 7). In parallel, StAR is phosphorylated by PKA, resulting in the full activity of the protein (11). To evaluate whether StAR and PKAR1α co-localize to the mitochondria, we transfected MA-10 cells with Star-FLAG and GFP-Pkar1α constructs and investigated the localization of these proteins in relation to mitochondria in triple labeling experiments using far-red MitoTracker (Fig. 3A). In control cells, there was a co-localization of StAR with the mitochondria but only a limited co-localization with PKAR1α. Ten minutes after the addition of 50 ng/ml hCG, there was a dramatic redistribution of the proteins and co-localization in the mitochondria in agreement with data shown above for PKAR1α localization to the mitochondria in response to hCG treatment (Fig. 2). This co-localization persisted for 30 min. Taken together, these results indicate that hormone treatment of Leydig cells results in the redistribution of PAP7 to TSPO-rich mitochondria where PAP7 binds to TSPO. The presence of PAP7 in the mitochondria leads to the transport of PKAR1α to this organelle, probably at the PAP7-TSPO sites. StAR protein is also targeted to the mitochondria because of its N-terminal mitochondrial signal sequence that interacts with both TSPO (16) and, most likely, the PKAR1α-PAP7-TSPO complex.

The interaction between PKAR1α and StAR was further confirmed in immunoprecipitation studies. Cells were transfected with or without GFP-STAR, GFP-TSPO, or GFP-Pkar1α constructs as described above. Cells were then treated with hCG for 10 min. Cell extracts were prepared and immunoprecipitated with an anti-GFP antibody. Proteins were separated on a native gel, transferred to nitrocellulose, and blotted with an anti-StAR antiserum. A nonspecific 220-kDa StAR immunoreactive complex present in the immunoprecipitates was seen in all samples and named “antibody complex” (Fig. 3B). A 240-kDa specific protein or protein complex immunoprecipitated by the GFP antibody was recognized by the StAR antiserum in cells transfected with GFP-Pkar1α and treated with hCG (Fig. 3B). Image analysis of the StAR-immunoreactive protein indicated a 5-fold induction by hCG (Fig. 3B, bar graph). A faint 240-kDa protein or protein complex was also immunoprecipitated by the GFP antibody and recognized by the StAR antiserum in cells transfected with GFP-TsPO and treated with or without hCG (Fig. 3B). Further immunoprecipitation studies accompanied by an analysis of the precipitated proteins by SDS-PAGE and immunoblotting failed to demonstrate a direct interaction between the proteins under investigation.

Identification of Protein-Protein and Protein-Cholesterol Interactions—Considering that the induction of steroidogenesis by hormones is a very rapid process, we favored the hypothesis that protein-protein interactions that mediate the acute response of the cells to hCG are short lived. We used a novel method using artificial amino acids, namely photoactivable leucine and methionine (30), to covalently cross-link interacting proteins and identify these interactions by immunoblotting after the separation of the proteins by SDS-PAGE. Using this method, we were able to identify a 220-kDa protein complex in COS1-F2–30 cells transfected with a Star construct and recognized by both anti-TSPO and anti-StAR antisera (Fig. 4A). A 240-kDa, TSPO-immunoreactive protein was seen in MA-10 cells incubated with photoactivable amino acids and exposed to UV light (Fig. 4B). The 18-kDa TSPO protein monomer was also identified as well as TSPO-immunoreactive proteins of...
FIGURE 2. Confocal microscopy images for subcellular localization of PKARI$\alpha$, TSPO, and PAP7. A, CFP/YFP microscopy in MA-10 cells. The green color staining in a, d, and g indicates the expression of pECFP-N1-Tspo. Constructs pEYFP-C1-Pap7, pEYFP-N1-Pap7, and pEYFP-N1-PkarI$\alpha$ were co-transfected with pECFP-N1-Tspo and are shown in red color in b, e, and h, respectively. c, f, and i represent the merged images of the data shown in a and b, d and e, and g and h, respectively. Co-localization of PKA-RI$\alpha$ with TSPO (B) or PAP7 (C) was examined in control and hCG-treated MA-10 cells. The cells were double stained for PKARI$\alpha$ and TSPO (j and m, anti-TSPO; k and n, anti-PKA-RI$\alpha$; i and o, merged images) or PKARI$\alpha$ and PAP7 (p and s, anti-PAP7; q and t, anti-PKA-RI$\alpha$; r and u, merged images). j, k, and i and p, q, and r show control cells; m, n, and o and s, t, and u show cells treated with 50 ng/ml hCG for 120 min. Similar results were obtained at 30-min hCG treatment (data not shown). Scale bar, 20 $\mu$m.
FIGURE 3. Interaction between PKAR\(\alpha\) and StAR. A, confocal microscopy images for subcellular localization of PKAR\(\alpha\) and StAR. Co-localization of PKAR\(\alpha\) and StAR in the mitochondrial was examined in control and hCG-treated MA-10 cells. Merged images are shown at the far right. Scale bar, 20 \(\mu\)m. B, MA-10 cells were transfected with pEGFP-Tspo, pEGFP-PkarI\(\alpha\), or empty vector. Forty-eight hours later, cells were washed and treated with or without 50 ng/ml hCG for 30 min. At the end of the treatment, cells were lysed and immunoprecipitated (IP) with anti-GFP antibodies. Samples were then incubated with protein G-Sepharose, precipitates were analyzed by non-reducing PAGE, and separated proteins were transferred to polyvinylidene difluoride membranes and immunoblotted (IB) with anti-StAR antibodies. Data (means \(\pm\) S.E.) obtained from three independent experiments were analyzed by image analysis. M, mock; C, control.
50–70 kDa, most likely representing TSPO homopolymers (15) or heteropolymers. Treatment of the cells with hCG (50 ng/ml) for 10, 30, or 120 min did not affect the amount of the 240-kDa, TSPO-immunoreactive protein. Because the size of the cross-linked protein (240 kDa) was identical to the protein immunoprecipitated and recognized by the TSPO, StAR, and PKAR1δ antisera (Fig. 3B), we investigated the presence of other proteins in the 240-kDa band. Fig. 4C shows the presence of PAP7, PKAR1δ, StAR, and outer mitochondrial membrane, voltage-dependent anion channel (VDAC1) immunoreactivities in the 240-kDa protein band. The ANT, an inner mitochondrial membrane protein, was not observed, even in overexposed blots. Interestingly all these proteins were present in the 240-kDa complex in control cells, although only minute amounts of StAR were found under these conditions (Fig. 4C). Treatment of MA-10 cells with hCG induced an increase in the amount of F2-130 cells, expression of Star induced the synthesis of pregnenolone 6.3- and 2.4-fold, respectively, compared with cells transfected with empty vector. Transfection of MA-10 cells with Star resulted in a 22-fold increase in progesterone. In both cell types, expression of Pap7 induced steroid formation to nearly the same extent as did Star. Transfection of Tspo in COS-F2-130 cells did not induce dramatic changes in pregnenolone formation, although it induced both pregnenolone and progesterone synthesis by 2.2- and 12-fold, respectively, in MA-10 cells. Expression of Pkar1 induced pregnenolone formation by 2.4- and 4.7-fold in MA-10 and COS-F2-130 cells, respectively, and the synthesis of progesterone by 15-fold in MA-10 cells.

Co-transfection of Tspo and Star plasmids resulted in an additive effect that led to a 3.2- and 8.7-fold induction of pregnenolone formation by MA-10 and COS-F2-130 cells, respec-
and a 10-fold induction of pregnenolone in COS-F2-130 cells, were obtained by transfecting Star together with Tspo, Pap7, and Pkarα proteins. Considering that these results are based on co-transfection studies where transfection efficiency plays a critical role, it is difficult to extrapolate the data obtained to steroid synthesis in response to hCG. However, it is noteworthy that co-transfection of all components of the proposed mitochondrial protein signaling complex resulted in robust steroid production in the absence of any hormonal stimulation even in cells lacking the luteinizing hormone receptor-G-protein signaling pathway (i.e. COS-F2-130 cells).

Next we investigated the effects of increasing concentrations of hCG on MA-10 cells co-transfected with or without the four genes of the complex. Fig. 5 (C and D) shows the dose-dependent formation of pregnenolone and progesterone in MA-10 cells. In the presence of submaximal concentrations of hCG (≤1.0 ng/ml), the effects of hCG were potentiated by the presence of the four genes. However, in the presence of maximal concentrations of hCG (≥10 ng/ml), the presence of the four genes decreased the amount of progesterone (48–67%) and pregnenolone (48–62%), respectively, compared with the treatments without transfection.

Because PAP7 has the ability to interact with Pkarα and a cell-permeable, PKA-anchoring inhibitor, the Ht31 peptide, derived from the PKA receptor-binding site of the human thyroid AKAP (34, 35) has been shown to inhibit Pkarα-AKAP interactions in various models, we examined whether this peptide could inhibit both the in situ reconstitution and activity of the STARP-Karα-PAP7-TSPO signaling complex and hCG-stimulated steroidogenesis. The peptide Ht31P, devoid of inhibitory activity, was used as a control. The concentrations of Ht31 and Ht31P used were optimized to 25 μM (data not shown). Fig. 6A shows that Ht31 inhibited 70% of hCG-stimulated steroid formation in a dose-dependent manner. Ht31, but not Ht31P, inhibited the four genes, inducing steroid formation by 60% (Fig. 6B). Moreover in the presence of saturating concentra-
tions of hCG, Ht31, but not Ht31P, produced a stronger inhibition of steroidogenesis.

The role of PAP7 in the induction of steroidogenesis was further confirmed in the following studies. An acyl-CoA-binding protein signature motif was found in the amino acid sequence of the PAP7 protein (18, 36). The acyl-CoA-binding protein motif is a structure that may bind to endogenous TSPO (37, 38). In addition, by comparing the amino acid sequence between PAP7 and D-AKAP1, we found that both mouse and human PAP7 proteins display homologies with the N0 isoform of D-AKAP1 within the first 30 amino acid residues (Fig. 6).

**Figure 6.** Effects of disruption of protein-protein interactions on steroidogenesis. A, effects of stearated Ht31 peptide on hCG-stimulated progesterone formation. MA-10 cells were treated with and without 25 μM Ht31 and Ht31P in the presence of increasing concentrations of hCG for 2 h. At the end of the incubation period, media were collected, and progesterone levels were determined by RIA. The effects of the co-transfection were statistically significant (p < 0.001) at all concentrations of hCG. The effects of Ht31, but not Ht31P, administered to cells transfected with the four genes were statistically significant (p < 0.001) compared with transfection alone. C, control. C, partial sequences (both cDNA and amino acid) of the N-terminal domain of mouse PAP7. The acyl-CoA-binding protein signature motif is underlined, and the shadowed amino acid sequence shows the PAP7 homologous region by comparison with the D-AKAP1 N0 isoform. D, alignment of the amino acid sequences of the N0 isoform targeting domain 1–30 with mouse (m) and human (h) PAP7 peptide residues. The shadowed amino acids show identical residues. E, effects of transfected N0 homologous and acyl-CoA-binding motif deletions in PAP7 on hCG-stimulated progesterone formation. Progesterone formation was measured 48 h after transfection. The boxed areas show the magnified pictures corresponding to the bars that indicate steroid formation under 0, 0.1, and 1 ng/ml hCG. The effect of co-transfection was statistically significant (p < 0.001) at all concentrations of hCG. The effects of mutant PAP7 was statistically significant (p < 0.001) compared with co-transfection with wild-type PAP7. C, control. In A, B, and E, results shown are means ± S.D. from three independent experiments performed in triplicate.
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targeting motif (22, 39). Fig. 6C shows the partial sequence of the PAP7 protein that contains the acyl-CoA-binding protein signature motif; the shaded domain shows the area of homology with the N0 isoform of D-ACKAP1. Based on the above sequence, we developed two PAP7 mutants: one with an acyl-CoA deletion and one with a deletion of the N0 homologous domain. The mutated genes were ligated into the mammalian expression vector pcDNA3.1(−) and used to replace the Pap7 gene in the four-gene co-transfection studies. Transfection of Star, Tspo, and PkarIα with the two mutated Pap7 sequences significantly decreased the formation of progesterone formed by cells under basal conditions and in the presence of saturating concentrations of hCG. In the absence of hCG, progesterone formation decreased by 83 and 90% in the presence of the acyl-CoA and N0 homologous PAP7 mutants, respectively. In the presence of 0.1–10 ng/ml hCG, progesterone formation decreased by 69–86% in the presence of the acyl-CoA PAP7 mutant and by 58–86% in the presence of the N0 homologous PAP7 mutant.

**DISCUSSION**

It is well established that hormonal activation of adenylate cyclase results in the synthesis of cAMP and that minimal amounts of cAMP induce a maximal rate of mitochondrial cholesterol transport and steroid synthesis. The mechanism underlying this amplification process remains unknown. Herein we provide evidence that this mechanism involves a mitochondrial macromolecular signaling complex that facilitates the transfer of cholesterol to Tspo and/or the initiation of the transfer of Tspo-bound cholesterol, taken up from either a cytosolic donor or storage sites, to the inner mitochondrial membrane through VDAC contact sites.

Initial evidence for the role of protein-protein interactions in hormone-induced steroidogenesis was presented by studying the spatial relationships of various proteins, i.e. Tspo, PAP7, and StAR, individually shown to be critical and necessary components of the steroidogenic machinery (12, 16, 17, 24–26).

The necessary role of PKARIα in mediating the action of cAMP also has been demonstrated (3–6). Live imaging of steroidogenic MA-10 and non-steroidogenic COS-F2-130 cells indicated that both Tspo and PKARIα are localized to the mitochondrial and cytoplasm, respectively, and that PAP7 is mainly found in the Golgi apparatus, although it is also present in cytosol and mitochondria, in agreement with previous findings (18, 36).

Immunofluorescence confocal microscopy confirmed these data. Co-transfection of YFP C- and N-terminal fusion proteins of PAP7 with a CFP N-terminal fusion protein of Tspo showed co-localization of the expressed proteins with the mitochondrial marker MitoTracker. The treatment of cells with hCG triggered a time-dependent movement of PAP7 from the trans-Golgi to the mitochondria, suggesting that the Golgi may be one of the primary targets of the hormone. Further studies suggested that hCG induces partial Golgi disassembly (data not shown) and that this event might be linked to increased steroid formation. Indeed treatment of steroidogenic cells with BFA increased the amount of PAP7 present in the mitochondria and potentiated hCG-induced steroidogenesis, suggesting that PAP7 release from the Golgi and its association with the mitochondria results in increased steroid formation. BFA withdrawal led to the reassembly of the Golgi apparatus. However, PAP7 was retained by the mitochondria, and steroid formation was maintained.

The finding that PAP7 was recruited to the Tspo-rich mitochondria from the trans-Golgi apparatus in a rapid cAMP-dependent manner suggests that such movement brings the PAP7 partner PKARIα (17) to the same mitochondrial sites. Indeed confocal microscopy demonstrated hormone-dependent targeting of PKARIα to the mitochondria. Interestingly hCG also induced the co-localization of PKARIα and StAR, confirmed by immunoprecipitation studies. Recent findings that StAR does not need to enter the mitochondria to exert its steroidogenic action (9, 10) and its association with proteins of the mitochondrial protein import system (10) suggested that StAR may interact with a mitochondrial acceptor/receptor. Although no direct Tspo-StAR interaction could be seen by yeast two-hybrid studies, the close relationship of these two proteins was observed by fluorescence energy transfer procedures (40), and their functional interaction in the support of steroid formation was recently demonstrated (16). Tspo is a high affinity, cholesterol-binding protein, integral to the outer mitochondrial membrane where it is functionally associated with VDAC (41), part of the outer/inner membrane contact sites (42), also enriched in Tspo (43). Taken together, these observations suggest that hormone-induced cAMP synthesis triggers protein redistribution that results in the targeting of PAP7 and PKARIα to Tspo in the mitochondria.

The lack of confirmation of certain visualized protein co-localization studies by immunoprecipitation, due to the short lived nature of the interactions, the weak interaction between the proteins, or the harsh conditions required to extract integral membrane proteins, such as Tspo, from the cells, drove us to experiment with novel approaches to identify protein-protein interactions in living cells. Because of their similarity to natural amino acids, photolucine and photomethionine are incorporated into proteins during translation (30). Cells with incorporated artificial amino acids were treated under various conditions and exposed to UV light, and the proteins were separated by SDS-PAGE and identified by immunoblot analyses. This methodology allowed us to identify an approximate 240-kDa protein complex in MA-10 cells that was recognized by anti-Tspo, -PAP7, -PKARIα, -STAR, and -VDAC1 antibodies. The absence of the inner mitochondrial membrane ANT from the complex, previously shown to also associate with Tspo (44), confirmed that this complex is formed at the outer mitochondrial membrane. Surprisingly this complex was present in control cells, although it contained very low levels of StAR immunoreactivity, in agreement with the presence of basal StAR transcription (45). Hormone treatment of the cells did not alter the size of the complex, but the amount of immunoreactive StAR present in the complex was increased in a time-dependent manner, reaching a maximum at 30 min. The time course of StAR association with the complex is similar to the time course of hCG-induced co-localization of StAR and

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PKAR1α in the mitochondria. The hCG-induced change in StAR levels present in the complex suggests that the composition of this complex changes although its molecular size remains the same; it is possible that size might be the limiting factor for its precise localization and/or function in the mitochondria. Considering that TSPO (18-kDa monomer, 54-kDa polymer), PAP7 (60 kDa), PKAR1α (54 kDa), and VDAC (32 kDa) are proteins found in many cells and that previous reports suggested that the native molecular size of TSPO isolated from mitochondria is close to 200 kDa (46, 47), the results presented here suggest the possibility that such a basal mitochondrial complex devoid of StAR may be present in various cell types. However, in steroidogenic cells, the presence of StAR brings hormone-response elements to the complex. It should be noted that despite the hCG-induced increase in PAP7 localization in the mitochondria and subsequent recruitment of PKAR1α to this organelle, we observed only a minor increase in these proteins in the 240-kDa complex. This may be due to the fact that these proteins are already present in the mitochondria under basal conditions and that a limiting amount of these proteins may participate in the complex; excess amounts may be stored and used during a later phase. VDAC1 levels were reduced following 2-h treatment with hCG. It has been shown that the ratio of TSPO to VDAC and ANT can be modulated by hormone treatments (48). However, the significance of this finding in steroidogenesis remains to be determined. Despite the increased presence of StAR in the 240-kDa complex in hormone-treated cells the size of the complex remains the same in both control and hormone-treated cells. Thus it can be assumed that the stoichiometry of the complex changes.

In search of the function of the complex in cholesterol transport, we investigated the physical interaction of cholesterol with the complex and examined the impact of co-expression of the StAR-PKAR1α-PAP7-TSPO proteins in steroidogenesis. In the later studies we did not include VDAC because this protein is a constitutive component of all mitochondria. Cells transfected with exogenous Tspo and incubated with photocholesterol followed by UV light exposure demonstrated recruitment of TSPO to the complex. Treatment of cells with hCG increased TSPO recruitment in the 240-kDa complex accompanied by increased levels of radioactivity in the samples, suggesting that the complex is part of the cholesterol transport machinery that delivers cholesterol into the mitochondria.

To investigate the individual role of each component of this signaling complex in steroidogenesis, we transfected the cDNAs of Pap7, Pkarr1α, Tspo, and Star either alone and in various combinations into MA-10 Leydig cells and COS-F2-130 monkey kidney cells. Expression of these cDNAs alone or in various combinations induced steroid formation in both MA-10 and COS-F2-130 cells. Maximal responses were obtained when the four cDNAs were co-transfected into the cells, reaching 10- and 50-fold increases in pregnenolone and progesterone formation by COS-F2-130 and MA-10 cells, respectively. For comparison, the hydrosoluble cholesterol compound (22R)-hydroxycholesterol induced COS-F2-130 pregnenolone formation 20-fold (data not shown), and saturating concentrations of hCG induced MA-10 progesterone synthesis 200-fold. This surprisingly robust stimulation of steroidogenesis, achieved in the absence of any exogenously supplied cholesterol, demonstrated the critical role of protein-protein interactions in steroid biosynthesis and suggested that targeting of these proteins to the right subcellular location could substitute for hormonal stimulation. These data also raised questions about the origin of the cholesterol used for steroid biosynthesis. It has long been proposed that cholesterol is stored in intracellular stores, such as lipid droplets and the plasma membrane, and that specific proteins could transport cholesterol to the mitochondria (2, 3, 5, 7). The present finding questions these proposals and presents the hypothesis that there is sufficient cholesterol in either the mitochondria or associated with the mitochondria to supply the initial steps in steroidogenesis. DiBartolomeis and Jefcoate (49) first proposed the existence of specialized steroidogenic pools of cholesterol in the mitochondria that are mobilized by hormone treatment and can support steroid synthesis for the first few minutes. Our data support this proposal in principal. However, it seems that these pools are sufficient to sustain steroidogenesis longer than just the first few minutes as their mobilization can be achieved by protein targeting to specific locations in mitochondria, and they do not seem to be specific to steroidogenic cells because COS-F2-130 cells behaved similarly. Maybe the identified mitochondrial signaling complex, or one or more of its components (TSPO?), might perform this cholesterol-sequestrating function that would allow for the presence of free cholesterol in the outer mitochondrial membrane.

Co-expression of PAP7, PKAR1α, TSPO, and StAR inhibited progesterone formation by MA-10 cells that had been stimulated with high concentrations of hCG (≥10 ng/ml), suggesting that these exogenous proteins may compete with the endogenously formed complex and act in a dominant-negative manner. The function of the endogenous mitochondrial signaling complex was assessed using Ht31. Ht31 inhibited hCG-stimulated steroid formation by 70%. Ht31 also reduced the efficacy of the four genes transfected into MA-10 cells. The fact that Ht31 did not completely abolish hCG-stimulated steroid synthesis may be due to the fact that this peptide was initially designed to block PKARIII-AKAP interactions. Nevertheless these data suggest that the PKAR1α-PAP7 interaction is critical for hormone-induced steroid synthesis.

Interestingly both RI and RII PKA have been shown to bind to D-AKAP1 amino acids 317–338, which form the R-binding domain (20). Further alignment results between the D-AKAP R-binding domain and PAP7 indicated that there are conserved amino acids present between AKAP and PAP7. These findings suggest that PAP7 might behave as a dual AKAP with preferential binding for RIα.

Previously we reported that overexpression of the partial PAP7 fragment, including the TSPO- and PKAR1α-binding domains, inhibited hCG-stimulated progesterone formation in Leydig cells, indicating that an overexpressed partial PAP7 fragment acts as a competitor of endogenous PAP7 and has a dominant-negative effect. In addition, overexpression of the partial PAP7 fragment did not alter hCG-stimulated cAMP accumulation, indicating that this dominant-negative effect is downstream of cAMP synthesis (17). We also know that PAP7 contains an acyl-CoA-binding protein signature motif and an N0
area homologous with D-AKAP1. Transfection of mutant Pap7 containing either acyl-CoA or N0 homologous deletions together with PkarIα, Tspo, and Star resulted in inhibition by 70–90% of hCG-induced steroid formation in MA-10 cells. The acyl-CoA signature motif, which is also found in the endogenous Tspo ligand DBI (37, 50), is the potential association site of Pap7 and Tspo. The N0 homologue area is another potential site of interaction of Pap7 with the mitochondria. The two homologous areas overlap at amino acid residues 86–94 (LALRFYKIK) of the Pap7 sequence, suggesting that this might be the domain that mediates the interaction between Pap7 and Tspo. This could also explain that, despite the presence of endogenous Tspo ligand DBI (37, 50), is the potential association site of Pap7 and Tspo. The N0 homologue area is another potential site of interaction of Pap7 with the mitochondria. The two homologous areas overlap at amino acid residues 86–94 (LALRFYKIK) of the Pap7 sequence, suggesting that this might be the domain that mediates the interaction between Pap7 and Tspo. This could also explain that, despite the presence of endogenous Tspo ligand DBI (37, 50), is the potential association site of Pap7 and Tspo. The N0 homologue area is another potential site of interaction of Pap7 with the mitochondria. The two homologous areas overlap at amino acid residues 86–94 (LALRFYKIK) of the Pap7 sequence, suggesting that this might be the domain that mediates the interaction between Pap7 and Tspo.

In conclusion, we observed the subcellular localization of Pap7, PkarIα, Tspo, and Star and their co-localization in response to hormone treatment, examined their functions in hCG-stimulated steroidogenesis, and investigated the effects of the disruption of the PkarIα-Pap7-Tspo interaction on steroid formation. The data obtained suggest that cAMP, and perhaps other intracellular signals initiated by hormones, regulate the level of a multivalent scaffold that resides on the cytosolic side of the outer mitochondrial membrane that assembles and integrates the cAMP signal, thus conferring cAMP signaling specificity, transduction, and amplification, and leads to both increased mitochondrial cholesterol transport and steroidogenesis.

In this identified complex, Pap7 acts as the signal transducer to bridge PkarIα and Tspo, and both the arrival of Star to the complex and subsequent formation of the Star-PkarIα-Pap7-Tspo mitochondrial complex provides its function in cholesterol movement. Thus, this scaffold allows the anchoring of PkarIα to the mitochondria and the recruitment of hormone-induced Star protein to this complex (Fig. 7). Although other mechanisms may participate in the hormonal induction of mitochondrial cholesterol transport and steroidogenesis, the finding that the disruptions of the PkarIα-Pap7 and Pap7-Tspo interactions led to a dramatic reduction in hCG-induced steroid synthesis suggests that these interactions are integral parts of the steroidogenic machinery. The formation, composition, and function of this complex in cholesterol transport and steroid formation, in the absence of hormonal stimuli or exogenous cholesterol, suggests that at least acute steroid production utilizes pools of cholesterol stored in or associated with the outer mitochondrial membrane. Additionally, cholesterol transfer to the inner mitochondrial membrane, where cholesterol metabolism occurs, appears to be mediated by protein-protein interactions in agreement with recent findings that Star acts outside the mitochondria to induce cholesterol transfer (9, 10) and that Tspo interacts with or mediates the action of Star (16).

Acknowledgments—We thank Dr. M. Ascoli (University of Iowa, Iowa City, IA) for the MA-10 Leydig cell line, Dr. W. L. Miller (University of California, San Francisco, CA) for the COS1-F2-130 cells, Dr. D. B. Hales (University of Illinois, Chicago, IL) for anti-Star antisera, Dr. C. Thiele (Max Planck Institute, Dresden, Germany) for the photo-activable artificial amino acids, Dr. M. Cully (Georgetown University) for critically reviewing the manuscript, and the National Hormone and Pituitary Program (NIDDK, National Institutes of Health) for the hCG.

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