Tyrosine phosphatase SHP2 regulates the expression of acyl-CoA synthetase ACSL4

Mariana Cooke,[1] Ulises Orlando,[1] Paula Maloberti, Ernesto J. Podestá, and Fabiana Cornejo Maciel[2]

Department of Biochemistry, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

Abstract **Acyl-CoA synthetase 4 (ACSL4) is implicated in fatty acid metabolism with marked preference for arachidonic acid (AA). ACSL4 plays crucial roles in physiological functions such as steroid synthesis and in pathological processes such as tumorigenesis. However, factors regulating ACSL4 mRNA and/or protein levels are not fully described. Because ACSL4 protein expression requires tyrosine phosphatase activity, in this study we aimed to identify the tyrosine phosphatase involved in ACSL4 expression. NSC87877, a specific inhibitor of the tyrosine phosphatase SHP2, reduced ACSL4 protein levels in ACSL4-rich breast cancer cells and steroidogenic cells. Indeed, overexpression of an active form of SHP2 increased ACSL4 protein levels in MA-10 Leydig steroidogenic cells. SHP2 has to be activated through a cAMP-dependent pathway to exert its effect on ACSL4. The effects could be specifically attributed to SHP2 because knockdown of the phosphatase reduced ACSL4 mRNA and protein levels. Through the action on ACSL4 protein levels, SHP2 affected AA-CoA production and metabolism and, finally, the steroidogenic capacity of MA-10 cells: overexpression (or knockdown) of SHP2 led to increased (or decreased) steroid production. We describe for the first time the involvement of SHP2 activity in the regulation of the expression of the fatty acid-metabolizing enzyme ACSL4.**—Cooke, M., U. Orlando, P. Maloberti, E. J. Podestá, and F. Cornejo Maciel. **Tyrosine phosphatase SHP2 regulates the expression of acyl-CoA synthetase ACSL4.** ***J. Lipid Res.*** **2011.** 52: **1936–1948.**

Supplementary key words protein tyrosine phosphatases • long-chain acyl-CoA synthetases • arachidonic acid • steroidogenesis • proliferation • Leydig cells • breast cancer cells

Acyl-CoA synthetases (ACS) are a family of enzymes that convert fatty acid to fatty acyl-CoA esters. Currently, five isoforms of the long-chain subfamily of ACS (ACSL; ACSL1, 3-6) have been identified and characterized in rodents and humans (1–5). These isoforms differ in their substrate preferences, enzyme kinetics, cellular and organelle locations, and regulation. ACSL4 has a marked preference for 20:4 (arachidonic acid, AA) and 20:5 (eicosapentaenoic acid). The high affinity of ACSL4 for these fatty acids and the low affinity for palmitic acid suggest that the enzyme plays a key role in the metabolism of AA. A second interesting feature of ACSL4 is its tissue distribution. In rats, its mRNA is expressed in various tissues, including the adrenal gland, epididymis, brain, lung, ovary, placenta, liver, and testis (6, 7). The striking feature of ACSL4 is its abundance in steroidogenic tissues, especially in *zona fasciculata* and *reticularis* of the rat adrenal gland, Leydig cells of the testis, and luteinized cells of the ovary (7). It is interesting that although relatively low or null expression levels of ACSL4 have been reported in other adult tissues (6, 7), this isoform is overexpressed in breast, prostate, colon, and liver cancer specimens (8–10).

Regarding its function, ACSL4 is related to the acute regulation of steroid production in steroidogenic tissues. ACSL4 is a key protein (11) that works in concert with a mitochondrial acyl-CoA thioesterase, ACOT2 (12, 13). These two fatty acid-metabolizing enzymes constitute an AA generation/export system, which releases AA in the mitochondrion after the action of the steroidogenic hormones adrenocorticotropin hormone (ACTH) and luteinizing hormone (LH)/chorionic gonadotropin (CG) (14). AA is then metabolized to lipoxygenated or epoxygenated products to induce the expression of the steroidogenesis acute regulatory (StAR) gene. StAR is a mitochondrial protein that, together with other proteins, participates in cholesterol transport to the inner mitochondrial membrane, which constitutes the rate-limiting

This work was supported by grants from University of Buenos Aires (UBA) to E.J.P. (M052) and F.C.M. (M409) and by Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) to E.J.P. (PICT 2006-00819). M.C. is the recipient of a CONICET fellowship. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Manuscript received 16 March 2011 and in revised form 27 August 2011.

Published, JLR Papers in Press, September 8, 2011
DOI 10.1194/jlr.M015552

Abbreviations: AA, arachidonic acid; ACSL4, acyl-CoA synthetase 4; ACTH, adrenocorticotropin hormone; BPA, benzylphosphonic acid; CG, chorionic gonadotropin; LH, luteinizing hormone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSC87877, 8-hydroxy-7-[(6-sulfo-2-naphthyl)azo]-5-quinolinesulfonic acid; PAO, phenyl arsine oxide; PKA, cAMP-dependent protein kinase; PTP, protein tyrosine phosphatase; SHP2, src homology 2-containing phosphotyrosine phosphatase 2; shRNA, short hairpin RNA; StAR, steroidogenic acute regulatory protein.

[1] M. Cooke and U. Orlando contributed equally to this work.
[2] To whom correspondence should be addressed.
e-mail: fcornejo@fmed.uba.ar

step of the steroidogenic biosynthetic pathway. Regarding the function of ACSL4 in other cell types, sustained downregulation of ACSL4 results in markedly reduced PGE2 release in human arterial smooth muscular cells (15), indicating that endogenous ACSL4 plays an important role as a regulator of eicosanoid synthesis and secretion, which might regulate smooth muscle cell proliferation, release of inflammatory mediators, or other processes in the vascular wall (15). ACSL4 also contributes to the development of human hepatocellular carcinoma, adenocarcinoma, and breast cancer (9, 10, 16, 17). ACSL4 is significantly upregulated in highly aggressive breast cancer cell lines (10, 17). The effect of ACSL4 on cell proliferation, invasion, and migration is attributed to increased production of lipoxygenase and cycloxygenase metabolites (17), which are known to potentiate tumor aggressiveness in hepatocellular carcinoma (8), colon adenocarcinoma (9), and breast cancer (10, 17). In summary, differences in the abundance and/or activity of ACSL4 may result in variations in the cellular content of free AA and AA-CoA, as well as in lipoxygenated and cycloxygenated metabolites. These compounds in turn play critical roles in steroid production in steroidogenic systems and in cell proliferation, migration, and invasion in cancer progression.

The cAMP-dependent action of ACTH and LH/CG on ACSL4 described above is mediated by the activity of protein tyrosine phosphatases (PTP) (18). The use of compounds that inhibit PTP enzymatic activity results in a decrease in cAMP-stimulated ACSL4 protein levels, in cAMP-stimulated steroid production (19–22), and in cAMP-stimulated tyrosine dephosphorylation of paxillin and focal adhesion disassembly (23). Although cAMP-dependent stimulation of steroidogenic cells increases the activity of several PTPs, the PTP(s) responsible for the increase in ACSL4 protein levels and steroid production is (are) still not identified. In this work, we focused on an 80 kDa hormone-activated PTP recognized by commercially available antibodies against src homology 2-containing phosphotyrosine phosphatase 2 (SHP2), a member of the nonreceptor PTP subfamily. In addition, SHP2 is phosphorylated in vitro by cAMP-dependent protein kinase (PKA) and in vivo by ACTH in bovine adrenal cells (24). In both cases, phosphorylation increases SHP2 activity. However, no evidence is available on the relationship between this activation and the steroidogenic function of the cell. SHP2 is an ubiquitously expressed nontransmembrane PTP that plays an essential role in many organisms, from lower eukaryotes to mammals (25, 26). Interestingly, SHP2 is one of the PTPs that promote the activation, rather than the downregulation, of intracellular signaling pathways (27, 28), serving multiple hormone receptors and participating in the regulation of mitogenic signaling, cell adhesion, and cell migration (28). In addition, accumulating evidence suggests that SHP2 might play a pivotal role in carcinogenesis. It is upregulated in breast cancer cells and in approximately 70% of infiltrating ductal carcinoma of the human breast, implying its involvement in breast oncogenesis (29). In contrast, inhibition of this PTP leads to reversion from mesenchymal to a normal breast epithelial phenotype (30).

Because of this background, we postulate that SHP2 could be the tyrosine phosphatase necessary for ACSL4 protein induction. The aim of this study was to analyze the involvement of SHP2 in the regulation of ACSL4 protein levels. Here, we introduce the notion that there are SHP2 -determined high levels of ACSL4 protein in MDA-MB231 cells and that SHP2 action stimulates proliferation of these cultured breast cancer cells. In addition, using plasmid-mediated gene transfer and RNAi-mediated gene silencing, we provide evidence for the first time that SHP2 has an obligatory role in steroid production by MA-10 cells, a mouse Leydig cell line, regulating the expression of ACSL4 protein, the rate-limiting enzyme in the AA generation/export system.

MATERIALS AND METHODS

Reagents

8Br-cAMP, arachidonic acid (AA), BSA, essentially free-fatty acid BSA, collagenase, Waymouth MB/752, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and phenylarsine oxide (PAO) were obtained from Sigma Chemical Co. Horse serum, DMEM, Medium 199, trypsin-EDTA, penicillin-streptomycin, and Lipofectamine 2000 were purchased from GIBCO-Invitrogen. FBS was obtained from PAA Laboratories. Bis-acetoxymethyl ester (AM) derivative of benzylphosphonic acid (BPA) [benzylphosphonic acid-$(AM)_2$, (BPA-$(AM)_2$)] was purchased from BIOMOL International, and 8-hydroxy-7-[(6-sulfo-2-naphthyl)azo]5-quinolinesulfonic acid (NSC87877) from Tocris Bioscience International. PVDF membrane, goat anti-rabbit, and anti-mouse IgG secondary antibody conjugated with horseradish peroxidase were obtained from Bio-Rad. Sterile and plastic material for tissue culture was from Orange Scientific. All other chemicals used in this study were of highest possible quality.

Cell cultures

MA-10 mouse Leydig tumor cells were generously provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA) and cultured in Waymouth MB/752 medium supplemented with 15% horse serum and antibiotics at 37°C and 5% CO_2 as previously described (31). The culture medium was replaced with serum-free Waymouth medium before experiments.

The human breast cancer cell line MDA-MB-231 was generously provided by Dr. Vasilios Papadopoulos (Research Institute of the McGill University Health Centre, Montreal, Canada) and obtained from the Lombardi Comprehensive Cancer Center (Georgetown University Medical Center, Washington, DC). The cell line was maintained in DMEM medium supplemented with 10% FBS and antibiotics at 37°C and 5% CO_2 as previously described (17).

Interstitial testicular cells were obtained from adult Wistar male rats. The animals were purchased from the animal facility of the School of Pharmacy and Biochemistry, University of Buenos Aires. The studies were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the School of Medicine, University of Buenos Aires. Freshly isolated interstitial cells were prepared by collagenase dispersion of testes as previously described (32). Cells were resuspended in Medium 199 containing 1.2 g/l $NaHCO_3$, and 0·5% BSA, at a density of 10^6 cells/ml and were maintained under a carbogen (95% O_2:5% CO_2) atmosphere throughout the procedures.

Plasmids and sequences

Mammalian expression vectors encoding the wild-type and the catalytically inactive SHP2 mutant, Addgene plasmids 8381 and

8382 (referred to as WT-SHP2 and CS-SHP2, respectively), were obtained from Addgene. CS-SHP2 is rendered catalytically inactive by the C459S mutation in its catalytic site. Both forms were obtained in the pRC/CMV plasmid. The amplification procedure was verified by nucleotide sequencing (Macrogen), and the obtained sequence was compared with that deposited in Genbank [*Homo sapiens* protein tyrosine phosphatase, nonreceptor type 11 (PTPN11), mRNA, accession number NM_002834].

To obtain the knockdown plasmid, we used a 19-bp DNA fragment of the mouse SHP2 (nucleotides 279-297) (33) in the adequate frameshift to generate a short hairpin RNA (shRNA), which the cells process to generate a functional and active siRNA directed against the murine SHP2. A BLAST search confirmed that the sequence specifically recognizes mouse SHP2. The insert was cloned in the pSUPER.retro.puro vector (Oligoengine). The obtained SHP2 shRNA construct was verified by nucleotide sequencing (Macrogen), and the obtained sequence was compared with that deposited in Genbank [*Mus musculus* protein tyrosine phosphatase, nonreceptor type 11 (Ptpn11), transcript variant 1, mRNA, accession number NM_011202]. Nontargeting control shRNA (named scrambled shRNA) consists of the same 19 nucleotides in a scrambled sequence, which does not target any known mouse cellular mRNA.

The knockdown of ACSL4 was performed with an already described pSUPER.retro plasmid containing ACSL4 shRNA (nucleotides 1809-1827) (17).

Transfections

For transient transfections, MA-10 cells were cultured in 6-well plates ($3.5\text{-}4 \times 10^5$ cells/well) overnight. The different constructions, WT-SHP2, CS-SHP2, SHP2 shRNA, scrambled shRNA, and ACSL4 shRNA, and pRC/CMV and pSUPER.retro.puro as respective controls, were transfected (4 μg DNA) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Overexpression and knockdown efficiency was confirmed by Western blot analysis using specific antibodies against SHP2. Overexpression of endogenous SHP2, monitored by immunoblot analysis with anti-SHP2 antibody, was confirmed in MA-10 transfected with the mammalian expression vectors 24 h posttransfection. Downregulation by shRNA occurred 48 h posttransfection of the pSUPER-retro plasmid.

Transfection efficiency for enhanced green fluorescent protein (EGFP) ranged from 30 to 45%. Neither up- nor downexpression of SHP2 affected cell viability, as assessed by the MTT assay. These results indicate that the transfection procedures do not affect mitochondrial function. Moreover, staining of the cells with the nuclear dye (DAPI) showed no change in nuclear morphology, indicating that none of the treatments induced apoptosis in MA-10 cells (data not shown).

Cell treatments

MA-10 cells were pretreated with or without NSC87877 (50 μM) for 3 h and then incubated in the presence or absence of 8Br-cAMP at the submaximal concentration of 0.5 mM for 1 h, with or without addition of AA (200 μM). Transfected MA-10 cells were incubated in the presence or absence of 8Br-cAMP at the submaximal concentration of 0.5 mM for the indicated times (see figure legends), with or without addition of AA (200 μM). AA treatments were performed in serum-free medium with 1% BSA fatty acid-free for 30 min prior to the stimulation. At the end of all experiments, progesterone production was determined in the culture media by RIA (21). MDA-MB-231 was treated with or without addition of PAO (1 μM) or BPA-(AM_2) (0.25 mM) (cell permeant derivative of the PTP inhibitor BPA) or NSC87877 (50 μM) for the indicated times (see figure legends). When steroid production was evaluated, testicular interstitial cells (10^6 cells) were pretreated with or without NSC87877 (50 μM) for 30 min and then incubated in the presence or absence of 8Br-cAMP 0.5 mM for 3 h at 37°C with gentle shaking. Testosterone production was determined in the incubation media by RIA (21). At the end of all experiments, MA-10, MDA-MB-231, and testicular interstitial cellular protein content were assayed by the Bradford method using BSA as standard (34).

PTP assay

SHP2 PTP activity was monitored by an in-gel PTP assay using [^{32}P]poly(Glu:Tyr) as substrate, with minor modifications (21) of the original description (35). Proteins exhibiting PTP activity are visualized as clear bands on a dark background due to removal of inorganic [^{32}P]phosphate from the radiolabeled substrate incorporated into the matrix gel (21, 35, 36).

Western blotting

SHP2 and ACSL4 protein levels were analyzed in a total cell lysate, and StAR protein levels were analyzed in the mitochondrial fraction. The primary antibodies used were purified monoclonal mouse antibody anti-SHP2 (BD-transduction laboratories), polyclonal rabbit antibody anti-ACSL4 (13, 37) (obtained at our laboratory), rabbit antisera anti-StAR (generously provided by Dr. Doug Stocco), anti-β-tubulin (used as cell lysate loading control, Millipore), and anti-Ox-Phos complex III core 2 subunit (used as mitochondrial loading control, Invitrogen). The secondary antibodies were goat anti-mouse or goat anti-rabbit immunoglobulins (according to the primary antibody used) conjugated to peroxidase. The bound primary antibodies were detected using an enhanced chemiluminescence kit (GE Healthcare). The levels of SHP2, ACSL4, StAR, β-tubulin, and complex III were quantitated using a computer-assisted image analyzer (ImageQuant 5.2), and the results for each sample were normalized by β-tubulin or complex III.

RNA extraction and semiquantitative RT-PCR

Total RNA from the different treatment groups was extracted using TriReagent following the manufacturer's instructions (MRC, Inc.). The reverse transcription and PCR analyses were made using 2 μg of total RNA. The cDNAs generated were further amplified by PCR under optimized conditions using the primer pairs below. For amplification of *Acsl4* (amplicon size 420 bp), the sense primer, 5′-GCCCACTTCAGACAAACCTGG-3′, and the antisense primer, 5′-ACAGCTTCTCTGCCAAGTGTGG-3′, were used. For amplification of *StAR* (amplicon size 566 bp), the sense primer, 5′-GGGACGAAGTGCTAAGTAAGATGG-3′, and the antisense primer, 5′-GGTCAATGTGGTGGACAGTCC-3′, were used. For amplification of *L19* ribosomal protein, used as housekeeping gene (amplicon size 405 bp), the sense primer, 5′-GAAATCGCCAATGCCAACTC-3′, and the antisense primer, 5′-TCTTAGACCTGCGAGCCTCA-3′ (38) were used. The reaction conditions were as follows: for *Acsl4* and *L19*, one cycle of 94°C for 5 min, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s, 94°C for 5 min, and one cycle of 72°C for 10 min; for *StAR*, one cycle of 94°C for 5 min, followed by 26 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and one cycle of 72°C for 10 min. The number of cycles was optimized for each gene to fall within the linear range of PCR amplification. PCR products were resolved on a 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide to determine the molecular sizes of the *Acsl4*, *StAR*, and *L19* amplicons. To compare the amounts of amplified *Acsl4* and *StAR* produced from different RNA samples, the amplified *L19* product of each sample was used as an internal standard. The gel images were acquired with the GelPro analyzer (IPS). The levels of *Acsl4*, *StAR*, and *L19* mRNA were quantitated

using a computer-assisted image analyzer (ImageQuant 5.2), and the PCR results for each sample were normalized by *L19* mRNA.

Cell proliferation assay

Cell proliferation was measured by the MTT and 5-bromo-2'-deoxyuridine assays.

The MTT assay, based on MTT reduction by the mitochondrial diaphorase enzyme, is an index of mitochondrial integrity (39). It was performed as previously described (17, 40). DNA synthesis was monitored by a colorimetric immunoassay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation (BrdU cell proliferation ELISA kit, Roche Diagnostics). The assay was performed according to the manufacturer's instructions.

[1-^{14}C]AA incorporation and AA-CoA measurement

MA-10 cells were labeled using [^{14}C]AA following a previously described methodology (41) with minor modifications (42). Briefly, [^{14}C]AA (New England Nuclear; specific activity 53.0 mCi/mmol) was incorporated (0.5 μCi/ml in serum-free Waymouth MB/752 containing 0.5% essentially fatty acid-free BSA) during 3 h at 37°C. The cells were incubated in the presence or absence of 0.5 mM 8Br-cAMP for 1 h, and cell lysates were obtained using a lysis buffer containing 20 mM Tris, pH 7.4, 0.5% Triton X-100, 1 mM EGTA, 1 mM EDTA, and 130 mM NaCl. Lipid extraction was performed twice with ethyl acetate (six volumes of solvent per one volume of cellular fraction) and centrifugation at 800 *g* for 5 min. The organic phase was discarded, and the [^{14}C]AA-CoA formation was evaluated by extraction from the aqueous phase as previously described (42, 43).

Statistical analysis

All experiments were run in duplicate and repeated at least three times. The reported data are means ± SEM of the three (or more) independent experiments. As appropriate, statistical analysis was performed by Student *t*-test (comparison of two data sets) or one-way ANOVA. The latter was followed by posttests if $P < 0.05$ by ANOVA, using the Instat software package from GraphPad, Inc.

RESULTS

Effect of inhibition of SHP2 on ACSL4 protein levels and cell proliferation in MDA-MB-231 breast cancer cells and on ACSL4 protein levels and steroidogenesis in MA-10 Leydig cells and testicular rat interstitial cells

To test whether SHP2 could be the PTP involved in the regulation of ACSL4 protein expression, we evaluated cell functions under SHP2 inhibition using NSC87877, a potent inhibitor of this PTP (44).

MDA-MB-231 cells derive from a highly aggressive human breast tumor and express high levels of ACSL4 mRNA and protein (10, 17). We used MDA-MB-231 cells to investigate the relationship between SHP2 and ACSL4 and cell proliferation. We first evaluated ACSL4 protein levels in cells where PTPs were inhibited. To this end, cell cultures were treated with or without PAO (1 μM), BPA-(AM_2) (0.25 mM), or NSC87877 (50 μM). While PAO and BPA act through two different mechanisms on all members of the PTP family, NSC87877 is a specific inhibitor of SHP2. The inhibition of PTPs with PAO or BPA reduced ACSL4 protein levels in breast cancer cells of the MDA-MB-231 cell line (**Fig. 1A**). NSC87877 also showed an inhibitory effect on ACSL4 protein levels (Fig. 1A), directly involving SHP2 in the induction of this acyl-CoA synthetase. Next, we measured the proliferation of MDA-MB-231 cells in the presence of NSC87877, which, because of the reduction in ACSL4 protein levels, should affect AA generation and metabolism. SHP2 inhibition with this compound resulted in a significant decrease in the number of MDA-MB-231 cells in the cultures at the end of the treatment, as determined by the MTT (Fig. 1B) and 8BrdU (Fig. 1C) assays.

We next used MA-10 Leydig cells to investigate the relationship between SHP2 and steroidogenesis. Cell cultures were treated with or without 8Br-cAMP (0.5 mM) in the presence or absence of NSC87877 (50 μM). ACSL4 protein level and progesterone production were determined under SHP2 inhibition. As previously described (11), we observed an increase in ACSL4 protein levels after the action of 8Br-cAMP (Fig. 1D). The SHP2 inhibitor abolished the increase in ACSL4 protein levels (Fig. 1D). Regarding steroidogenesis, cAMP-stimulated progesterone production was reduced by the use of NSC87877 (Fig. 1E).

To demonstrate that the effect of NSC87877 on steroidogenesis is due to the reduction in ACSL4, we exogenously added AA. We previously published that the cAMP-dependent signal transduction pathway activates tyrosine phosphatases upstream of ACSL4/Acot2 action (18) ACSL4 is an enzyme involved in the generation and export of mitochondrial AA (11, 17, 42). ACSL4 converts AA into the CoA ester, which is then the substrate for Acot2, a mitochondrial thioesterase. Because of the sequential action of these two enzymes, there is a compartmentalized generation of AA (12, 13) and metabolism through the lipoxygenase pathway (17). Therefore, the addition of AA should by pass the inhibition of SHP2 on steroidogenesis. As was already known, AA increased steroid production by itself and with cAMP (42). Together with NSC87877, AA blunted the inhibition exerted by NSC87877 on cAMP-stimulated progesterone production (Fig. 1E). In conclusion, the result obtained with the exogenous addition of AA indicates that the inhibition observed with NSC87877 reinforces the notion that SHP2 would be acting on AA generation because of its inducing action on ACSL4.

Because we used cell lines, we questioned whether the observed effects also occurred in a primary cell culture. Thus, we tested the effect of SHP2 inhibition on Leydig cells by incubation of interstitial rat testis cells with NSC87877, followed by evaluation of ACSL4 protein level and testosterone production upon stimulation by 8Br-cAMP. The 8Br-cAMP treatment caused an increase in ACSL4 protein levels (Fig. 1F) and testosterone production (Fig. 1G), as expected. In the presence of the SHP2 inhibitor, there were lower levels of ACSL4 protein and testosterone production after cAMP stimulation. These results confirmed the conclusions we reached using cell lines and validated using MA-10 Leydig cells as experimental model throughout the study.

As previously reported, ACSL4 was detected as a duplet (1, 17, 45, 46). This duplet may be seen in the three cell

types used. However, the difference between the two bands is better seen in MDA-MB-231 cells (Fig. 1A) than in MA-10 cells (Fig. 1B) or freshly isolated interstitial testis cells (Fig. 1F), where the electrophoresis conditions have to be finely adjusted to evidence the double band. Both alternative splicing and posttranslational modification mechanisms can explain the detection of a duplet by Western blots. The gene encoding ACLS4 shows alternative splicing (45, 46), and the protein is possibly acetylated. It was also suggested that the different mobility is due to proteolisis or posttranslational modifications, such as N-glycosilation (46).

The results obtained with NSC87877 assured us that SHP2 could be one of the PTPs involved in the increase in ACSL4 protein levels. This first approach encouraged us to further analyze the effect of the modification of SHP2 expression levels on the regulation of ACSL4 mRNA and/or protein expression and function in one of the cell types, the steroidogenic MA-10 Leydig cells. For that purpose, we used constructions with sequences that either upregulate or downregulate the amount of the endogenous enzyme.

Overexpression and knockdown of SHP2 in MA-10 Leydig cells

We first overexpressed SHP2 in MA-10 cells by means of a transient transfection approach using a pRC/CMV plasmid containing WT- or CS-SHP2 cDNA. The transfection of these plasmids renders the wild-type and catalytically inactive forms, respectively. Then, 24 h after transfection, SHP2 expression levels in basal and cAMP-stimulated conditions were evaluated by Western blot in the cell lysates. As expected, in basal nonstimulated conditions, transfection of both SHP2 cDNAs increased the SHP2 protein signal compared with the endogenous expression (**Fig. 2A**). Transfected WT-SHP2 migrated at the expected molecular weight of 80 kDa in SDS-PAGE and showed conserved PTP activity as evidenced by in-gel PTP assays (Fig. 2B). A higher intensity of PTP activity in a band of 80 kDa was obtained in WT-SHP2-tranfected cells compared with the mock-transfected cells. Expectedly, cells overexpressing CS-SHP2 showed PTP activity levels comparable to those of mock-transfected cells (Fig. 2B).

Regarding the effect of cAMP on SHP2, neither the SHP2 protein level of mock-transfected cells nor that of WT- or CS-SHP2-transfected cells was affected by treatment with cAMP (Fig. 2A). However, the stimulation of the cells with cAMP resulted in an increase in the enzymatic activity of an 80 kDa band in mock- and WT-SHP2-transfected cells (Fig. 2B). Transfection with the WT form of SHP2 displayed a basal activity that could be further increased by cAMP.

Next, we knocked down the expression of SHP2 using SHP2 shRNA. After transfection of SHP2 shRNA sequences, the efficacy of SHP2 targeting siRNA was confirmed by Western blot analysis (Fig. 2C) and in-gel PTP activity analysis (Fig. 2D). As expected, expression of SHP2-targeting shRNA decreased the level of endogenous SHP2, compared with both, empty, or scrambled shRNA transfections (Fig. 2C). A lower intensity of PTP activity in a band of 80 kDa was obtained in SHP2 shRNA-transfected cells (Fig. 2D).

To analyze the effect of modifying SHP2 protein levels on the final response of steroidogenic stimulation, we collected media from cells transfected with the constructs described above and determined steroid secretion. Overexpression of WT-SHP2 significantly increased cAMP-stimulated progesterone production compared with mock-transfected cells (Fig. 2E), whereas the expression of the catalytically inactive form caused a consistent and repeated slight decrease (but not statistically significant) in cAMP-stimulated steroidogenesis. The effect could be attributed specifically to SHP2, as MA-10 cell cultures transfected with SHP2-targeting shRNA, which downexpresses SHP2, significantly decreased cAMP-stimulated progesterone production compared with empty vector or scrambled shRNA transfections (Fig. 2F). Because empty vector or scrambled shRNA-transfected cells exhibited similar SHP2 protein levels and basal and cAMP-stimulated steroid production, both may be used as mock transfections.

Effect of overexpression and knockdown of SHP2 on ACSL4 expression in MA-10 Leydig cells

ACSL4 expression was analyzed in both conditions of SHP2 expression levels and the results are shown in **Fig. 3**. Mock transfection shows the already known increase in ACSL4 protein level under cAMP stimulation. WT-SHP2 overexpression resulted in a significant increase in ACSL4 protein levels as compared with mock-transfected cells, an effect potentiated by cAMP, while the levels of ACSL4 were unaffected by CS-SHP2 transfection. Furthermore, CS-SHP2 overexpression blunted the effect of cAMP on ACSL4 protein expression (Fig. 3A).

The expression of ACSL4 was also analyzed in cells where the expression of SHP2 was down-regulated. The reduced expression of SHP2 resulted in a significant decrease in the mRNA and protein levels of the synthetase (Fig. 3B–D). *Acsl4* mRNA level was increased in MA-10 Leydig cells treated with cAMP, an effect impaired by SHP2 shRNA (Fig. 3B). As in the previous experiment, cAMP increased ACSL4 protein expression in MA-10 Leydig cells, whereas SHP2 knockdown by shRNA abolished the effect, compared with mock (Fig. 3C) or scrambled shRNA (Fig. 3D) transfections. The decrease in ACSL4 protein levels in SHP2-down-regulated cells should also be reflected on the synthetase activity. To determine the endogenous activity of ACSL4, we measured AA-CoA production in MA-10 cells labeled with [1-^{14}C] AA. The cell cultures were labeled with radioactive AA, which, once incorporated, is the substrate of the endogenous ACSL4 that renders AA-CoA. The radioactive form of this derivative was determined after organic extraction. We reproduced the previously published stimulatory effect of cAMP on the content of the CoA derivative of AA (42), an effect impaired in SHP2-lacking cells (Fig. 3E).

We also analyzed the effect of SHP2 expression on ACOT2 protein levels, the second enzyme involved in AA generation (13). The results indicate that neither the overexpression nor the silencing of SHP2 affected ACOT2 protein levels (data not shown).

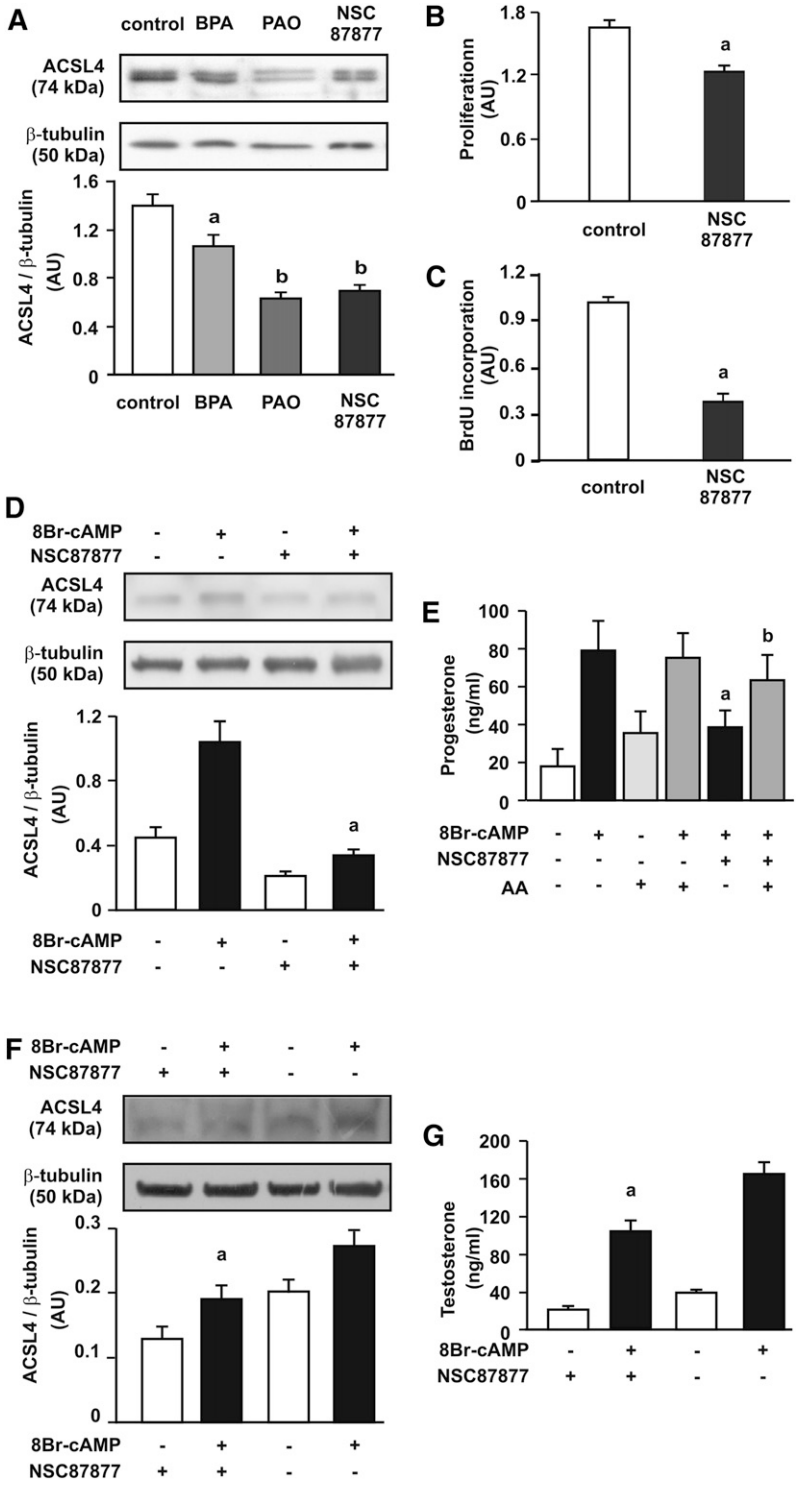

Fig. 1. Inhibition of SHP2 in MDA-MB-231 breast cancer cells, MA-10 Leydig cells, and interstitial rat testis cells: effect on ACSL4 protein levels, cell proliferation, and steroidogenesis. A: MDA-MB-231 cells were treated with PTP inhibitors, BPA-(AM_2) (0.25 mM), PAO (1 μM), or NSC87877 (50 μM) for 24 h. Cells were collected and the cell lysate proteins were used to analyze ACSL4 expression by Western blot with specific antibodies. A representative immunoblot is shown. The approximate molecular mass of each immunoblotted protein is indicated. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated, ACSL4 values were normalized against the corresponding β-tubulin band, and the results were expressed in arbitrary units (AU). The results are means ± SEM of three independent experiments. $^{a}P < 0.05$; $^{b}P < 0.01$ versus control. B and C: MDA-MB-231 cells were treated with NSC87877 (50 μM) for 96 h. Cell proliferation was measured by the MTT (C) and BrdU incorporation (D) assays. Data represent means ± SEM of three independent experiments. B: $^{a}P < 0.05$ versus control. C: $^{a}P < 0.001$ versus control. D: MA-10 Leydig cells were pretreated with or without NSC87877 (50 μM) for 3 h. The

Since the changes in ACSL4 protein levels and activity should be reflected in downstream steps such as StAR protein induction, we next examined the effect of overexpression and knockdown of SHP2 on StAR protein.

Effect of overexpression and knockdown of SHP2 on StAR expression in MA-10 Leydig cells

As StAR abundance depends on both the induction of ACSL4 protein and the generation of AA (11), we continued analyzing StAR protein levels in SHP2 up- or downregulated MA-10 Leydig cells. The results are shown in **Fig. 4**. The overexpression of WT-SHP2 significantly increased the expression of basal StAR protein compared with mock-transfected cells (Fig. 4A). Treatment with cAMP further increased the expression of StAR protein in the WT-SHP2-transfected cells. Overexpression of CS-SHP2 did not change StAR basal protein levels and impaired the stimulation produced by cAMP treatment.

Downregulation of SHP2 also modified StAR expression. Regarding *StAR* mRNA levels, StAR expression was increased in MA-10 Leydig cells treated with cAMP. This effect was impaired by SHP2 shRNA (Fig. 4B). Again, cAMP treatment of the cells caused a significant increase in StAR protein levels in mock-transfected cells, whereas SHP2 shRNA treatment prevented the effect of cAMP on StAR protein in MA-10 cells (Fig. 4C). The involvement of AA in this process was reinforced by the fact that the AA addition to SHP2 shRNA-treated cells bypassed the inhibitory effect described above (Fig. 4C).

Role of ACSL4 in SHP2-mediated effect on cAMP-stimulated steroidogenesis in MA-10 Leydig cells

There is a correlation between SHP2 and ACSL4 protein levels (Fig. 3). As stated above (Figs. 1E and 4C) and according to previous publications, ACSL4 action in steroidogenic cells directs AA to the compartamentalized metabolism through the lipoxygenase pathway. Thus, the observed inhibitory effect of SHP2 shRNA on steroidogenesis should be bypassed by the addition of AA. Indeed, AA was able to restore the steroid production in SHP2 shRNA-treated cells (**Fig. 5A**). The effects of AA alone or together with cAMP on mock-transfected cells reproduced previously published results (42).

Finally, additional demonstration of a role for ACSL4 in SHP2-mediated effects was obtained from siRNA experiments to knockdown ACSL4 expression in SHP2 overexpressing cells. Cotransfection of MA-10 Leydig cells with the plasmid that overexpresses the wild-type form of SHP2, together with a plasmid that silences ACSL4, exerted an inhibitory effect on cAMP-stimulated progesterone production and ACSL4 protein levels (Fig. 5B, C). Progesterone production by SHP2 overexpression in ACSL4-depleted cells was only partially inhibited, possibly due to incomplete knockdown of ACLSL4. The effect of an excessive expression of SHP2 could not be inhibited totally by the ACLS4 shRNA transfection.

DISCUSSION

The intracellular abundance of ACSL4 protein has been determined in physiological and pathological conditions, demonstrating changes under the action of several factors: fasting/feeding state (6), ACTH/cAMP (11, 47), and AA action (47), and transformation processes (9, 10, 16). The main finding of the present study is the identification of SHP2 as a PTP involved in the regulation of the expression of this previously known AA-metabolizing enzyme in highly proliferative breast cancer cells and in steroidogenic differentiated Leydig cells. This conclusion is based on two facts: *i*) overexpression and downregulation of SHP2 in a steroidogenic system such as MA-10 Leydig cells resulted in the upregulation and downregulation of ACSL4, respectively, and *ii*) inhibition of SHP2 in a proliferative system such as MDA-MD-231 breast cancer cells reduced ACSL4 expression.

The joint action of SHP2 and ACSL4 could regulate a physiological function such as steroid production or lead to a pathological process such as cell transformation. Regulation of ACSL4 might change the subcellular pools of AA and AA-CoA and thereby alter a broad range of cellular

stimulation was performed with or without 8Br-cAMP (0.5 mM) for 1 h. Cells were collected and the cell lysate proteins were used to analyze ACSL4 expression by Western blot with specific antibodies. A representative immunoblot is shown. The approximate molecular mass of each immunoblotted protein is indicated. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated, and ACSL4 values were normalized against the corresponding β-tubulin band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. $^{a}P < 0.001$ versus cAMP alone. E: MA-10 Leydig cells were pretreated with or without NSC87877 (50 μM) for 3 h and then incubated in the presence or absence of AA (200 μM). The stimulation was performed with or without 8Br-cAMP (0.5 mM) for 1 h. Steroid production was evaluated determining progesterone production in the culture media by radioimmunoassay. Data represent means ± SEM of five independent experiments. $^{a}P < 0.001$ versus cAMP alone; $^{b}P < 0.05$ versus cAMP + NSC87877. F, G: Interstitial testicular cells were pretreated with or without NSC87877 (50 μM) for 30 min. The stimulation was performed with or without 8Br-cAMP (0.5 mM) for 3 h. F: Cells were collected and the cell lysate proteins were used to analyze ACSL4 expression by Western blot with specific antibodies. A representative immunoblot is shown. The approximate molecular mass of each immunoblotted protein is indicated. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated, and ACSL4 values were normalized against the corresponding β-tubulin band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. $^{a}P < 0.05$ versus cAMP alone. G: Steroid production was evaluated determining testosterone production in the culture media by radioimmunoassay. Data represent means ± SEM of three independent experiments. $^{a}P < 0.01$ versus cAMP alone.

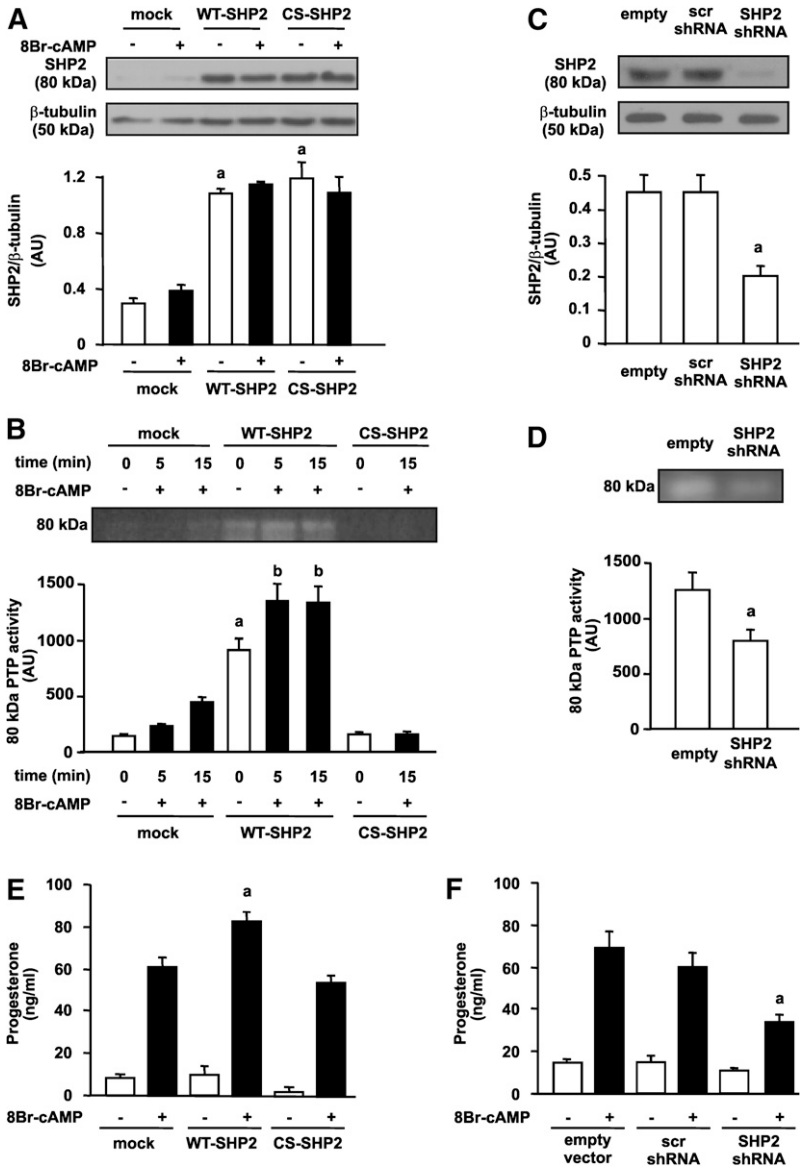

Fig. 2. Overexpression and knockdown of SHP2 in MA-10 Leydig cells: SHP2 protein levels, PTP activity, and steroid production. MA-10 cells were cultured and transiently transfected with an empty pRC/CMV (mock) or a pRC/CMV plasmid containing either a wild-type (WT-SHP2) or a catalytically inactive form (CS-SHP2) of SHP2 for 24 h (A, B, E) or with an empty pSUPER.retro.puro vector (empty) or a pSUPER.retro.puro vector containing scrambled shRNA (scr shRNA) or SHP2 shRNA (C, D, F) for 48 h. A and B: Cells were treated with or without 0.5 mM 8Br-cAMP for 1 h (A) or the indicated times (B). The cell lysates were used to analyze SHP2 protein levels by Western blot (A) or PTP activities by in-gel PTP assay (B). C and D: Cells were collected, and the cell lysate proteins were used to analyze SHP2 levels by Western blot (C) or PTP activity by in-gel PTP assay (D). A and C show representative immunoblots. The approximate molecular mass of each immunoblotted protein is indicated. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated. SHP2 values were normalized against the corresponding β-tubulin band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. A: $^{a}P < 0.001$ versus mock. C: $^{a}P < 0.05$ versus empty or scrambled shRNA containing vector. B and D show the autoradiography of a representative ^{32}P-poly (Glu:Tyr)-containing gel processed to develop PTP activity. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated. The values of the 80 kDa PTP activity are expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. B: $^{a}P < 0.001$ versus mock without cAMP; $^{b}P < 0.01$ versus WT-SHP2 without cAMP. D: $^{a}P < 0.05$ versus mock. E and F: MA-10-transfected cells were incubated in serum-free medium in the presence or absence of 0.5 mM 8Br-cAMP for 1 h. Steroid production was evaluated by determining progesterone concentrations in the medium. Data are shown as progesterone concentration in nanograms per milliliter. The results are means ± SEM of four independent experiments. E: $^{a}P < 0.01$ versus mock + cAMP. F: $^{a}P < 0.05$ versus empty + cAMP or scramble shRNA + cAMP.

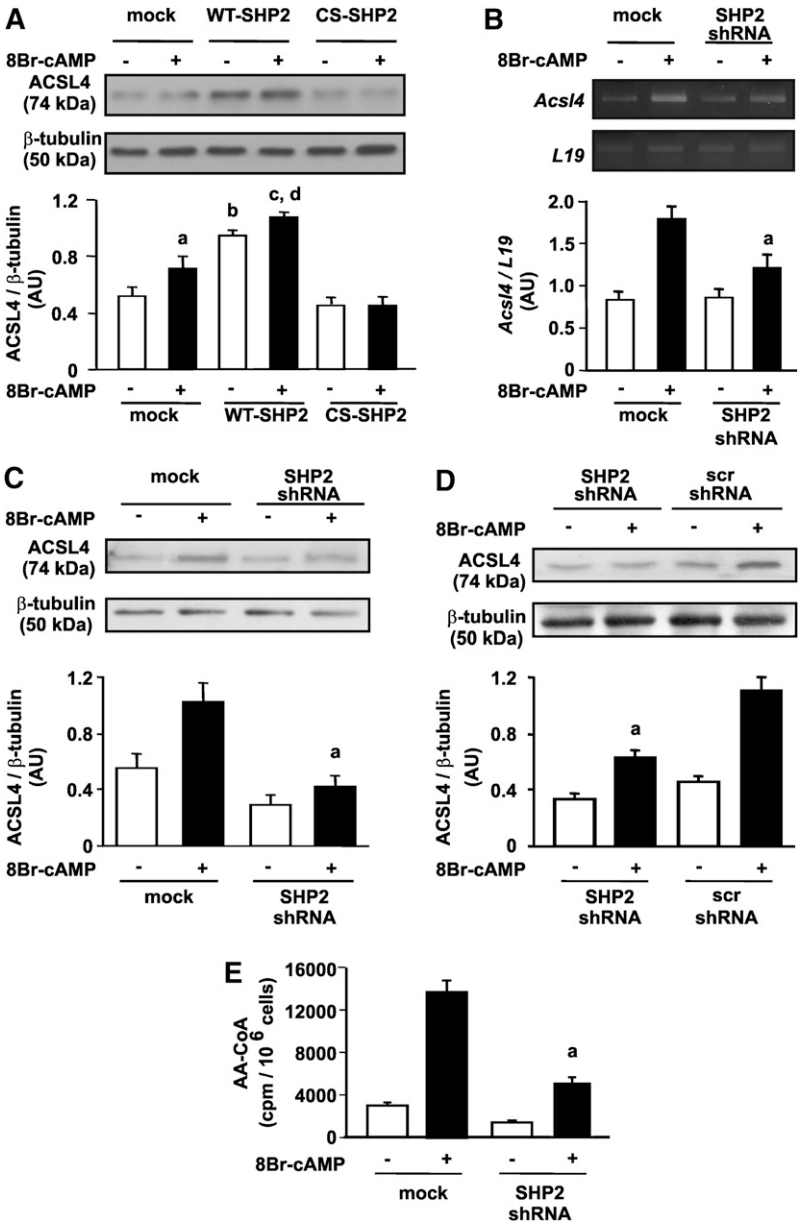

Fig. 3. Overexpression and knockdown of SHP2 in MA-10 Leydig cells: effect on ACSL4 expression and activity. MA-10 cells were cultured and transiently transfected with an empty pRC/CMV (mock) or a pRC/CMV plasmid containing either a wild-type (WT-SHP2) or a catalytically inactive form (CS-SHP2) of SHP2 for 24 h (A) or with an empty pSUPER.retro.puro vector (mock) or a pSUPER.retro.puro vector containing scrambled shRNA (scr shRNA) or SHP2 shRNA (B–E) for 48 h. A, C, and D: MA-10-transfected cells were incubated in serum-free medium in the presence or absence of 0.5 mM 8Br-cAMP for 1 h. Cells were collected, and the cell lysate proteins were used to analyze ACSL4 expression by Western blot with specific antibodies. Representative immunoblots are shown, and the approximate molecular mass of each immunoblotted protein is indicated. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated. ACSL4 values were normalized against the corresponding β-tubulin band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. A: $^{a}P < 0.01$ versus mock; $^{b}P < 0.001$ versus mock; $^{c}P < 0.001$ versus mock + cAMP; $^{d}P < 0.05$ versus WT-SHP2. C: $^{a}P < 0.001$ versus mock + cAMP. D: $^{a}P < 0.01$ versus scrambled shRNA + cAMP. B: MA-10-transfected cells were incubated in serum-free medium in the presence or absence of 0.5 mM 8Br-cAMP for 1 h. Total RNA was isolated and used in semiquantitative RT-PCR with specific primers for *Acsl4* cDNA and *L19* cDNA as loading control. PCR products were resolved in ethidium bromide-stained agarose gels. A representative gel is shown. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated. *Acsl4* values were normalized against the corresponding *L19* band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. $^{a}P < 0.01$ versus mock + cAMP. E: MA-10-transfected cells were labeled with [^{14}C]AA as described in "Materials and Methods" and incubated in serum free-medium in the presence or absence of 0.5 mM 8Br-cAMP for 1 h. Cells were collected, and the cell lysates were used to analyze AA-CoA accumulation by lipid extraction as described. The [^{14}C]AA-CoA content is expressed as means ± SEM of three independent experiments in cpm per 10^6 cells. $^{a}P < 0.001$ versus mock + cAMP.

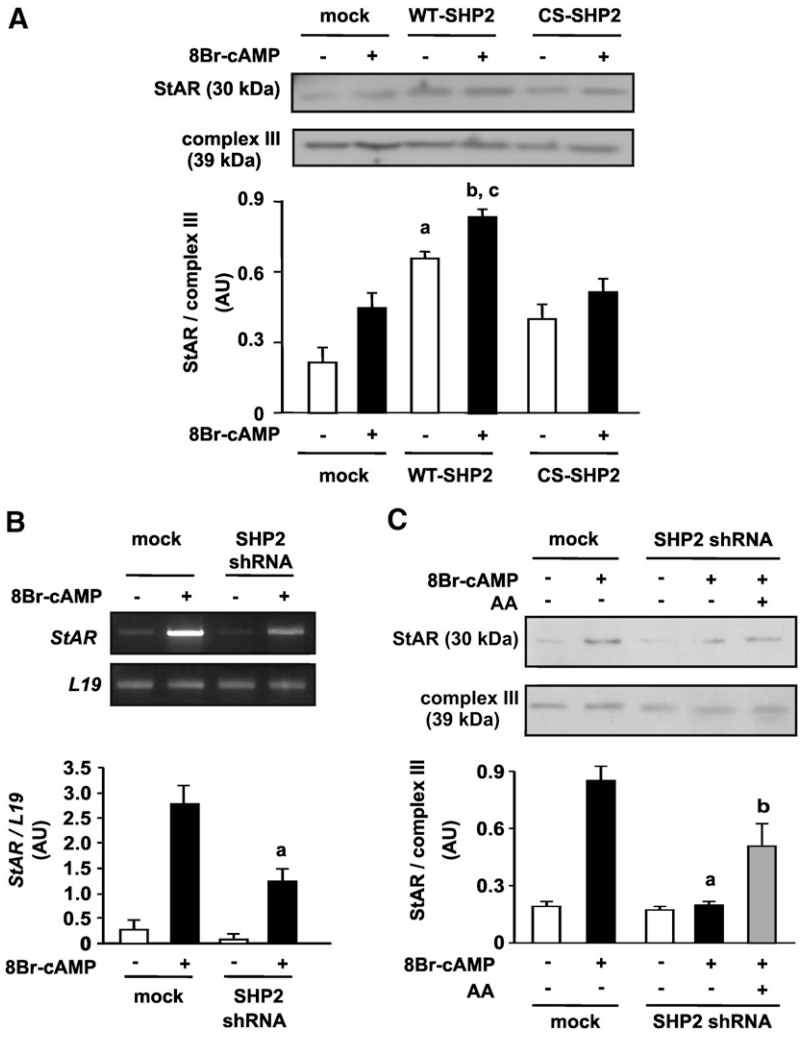

Fig. 4. Overexpression and knockdown of SHP2 in MA-10 Leydig cells: effect on StAR expression. MA-10 cells were cultured and transiently transfected with an empty pRC/CMV (mock) or a pRC/CMV plasmid containing either a wild-type (WT-SHP2) or a catalytically inactive form (CS-SHP2) of SHP2 for 24 h (A) or with an empty pSUPER.retro.puro vector (mock) or a pSUPER.retro.puro vector containing SHP2 shRNA for 48 h (B and C). A and C: MA-10-transfected cells were incubated in serum-free medium in the presence or absence of 0.5 mM 8Br-cAMP for 2 h. In C, the cells were previously incubated in the presence or absence of 200 μM AA. StAR protein levels were evaluated in the mitochondrial fraction by Western blot analysis. Representative immunoblots are shown, and the approximate molecular mass of each immunoblotted protein is indicated. Each experiment was performed three times with similar results. The integrated optical density of each band was quantitated. StAR values were normalized against the corresponding mitochondrial complex III bands and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. A: $^{a}P < 0.001$ versus mock; $^{b}P < 0.001$ versus mock + cAMP; $^{c}P < 0.05$ versus WT-SHP2 alone. C: $^{a}P < 0.001$ versus mock + cAMP; $^{b}P < 0.05$ versus SHP2 shRNA + cAMP. B: MA-10-transfected cells were incubated in serum-free medium in the presence or absence of 0.5 mM 8Br-cAMP for 1 h. Total RNA was isolated and used in semiquantitative RT-PCR with specific primers for *StAR* cDNA and *L19* cDNA as loading control. PCR products were resolved in ethidium bromide-stained agarose gels. A representative gel is shown. The integrated optical density of each band was quantitated. *StAR* values were normalized against the corresponding *L19* band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments. $^{a}P < 0.01$ versus mock + cAMP.

processes that are controlled by this ratio. Here we report that changes in ACSL4 protein levels altered AA-mediated cell functions: steroidogenesis in MA-10 Leydig cells and proliferation in MDA-MD-231 cells. As a result of ACSL4 upregulation, overexpression of SHP2 increased StAR protein in MA-10 Leydig cells. SHP2 has to be present and activated by a cAMP-dependent pathway as the overexpression of a catalytically inactive form of the enzyme (CS-SHP2) did not reproduce the stimulatory effects of the wild-type form on ACSL4 protein levels, and it reduced cAMP action on steroidogenesis. Moreover, the catalytically inactive form slightly reduced the basal levels of progesterone production. This was probably due to an already described substrate-trapping action of the CS-SHP2 mutant (48), which would reduce the action of the endogenous enzyme.

The overexpression of the wild-type form of SHP2 increased the levels of ACSL4 (Fig. 3) and StAR (Fig. 4) proteins in nonstimulated MA-10 cultures. However, there was no stimulatory effect on the basal steroid production (Fig. 2). These results are in line with previous publications that indicate that steroidogenesis is dependent on the activity of PKA (49, 50) and on phosphorylation and activation of StAR by PKA (51) and ERK (52). The sole increase in SHP2 and ACSL4 levels was not enough to increase steroid production because PKA and ERK activation are lacking.

The effects observed under overexpression of SHP2 could be due to high levels of this PTP unspecifically replacing the function of another endogenous PTP. This possibility was ruled out using NSC87877, a specific inhibitor of SHP2, and the SHP2-specific shRNA. The results confirmed the need for an endogenous SHP2 to regulate the levels of ACSL4 and StAR proteins and steroidogenesis. This is the first demonstration that SHP2 upregulates the levels of ACSL4, the rate-limiting enzyme of the AA generation/export system in mitochondria of steroidogenic cells. As AA is necessary for StAR protein induction and, finally, for steroidogenesis, this statement is reinforced by the fact that the effect of SHP2 shRNA on StAR and steroidogenesis was overcome by exogenous addition of AA. We propose that SHP2-mediated production of progesterone involves ACSL4. Indeed, silencing ACSL4 from MA-10 Leydig cells significantly reduced progesterone production in response to cAMP. Progesterone production by SHP2 overexpression in ACSL4-depleted cells is partially inhibited, possibly due to incomplete knockdown of ACLSL4 (Fig. 5B, C).

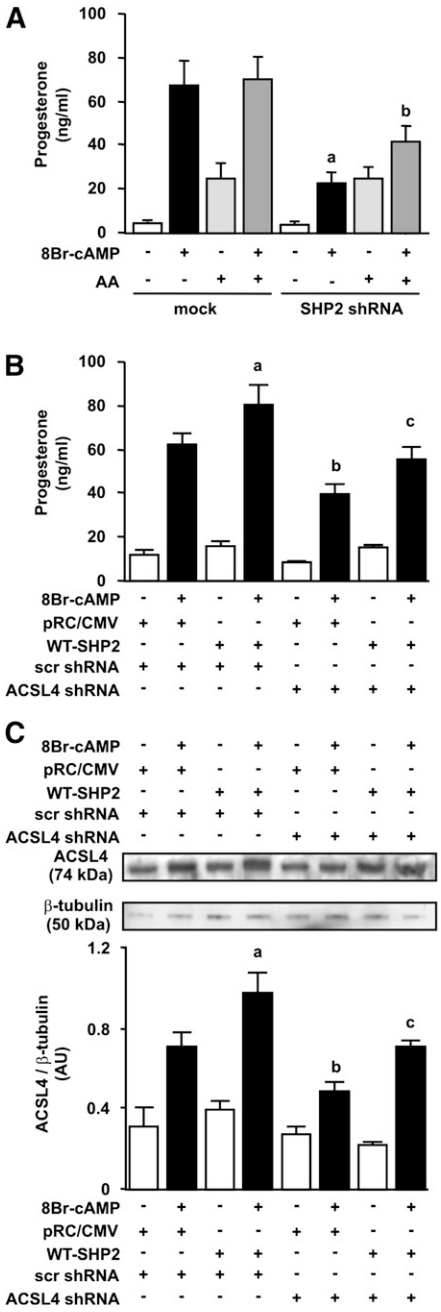

Fig. 5. Effect of AA on SHP2 shRNA inhibited steroidogenesis and effect of ACSL4 shRNA on WT- SHP2 stimulated steroidogenesis. MA-10 cells were cultured and transiently transfected with an empty pSUPER.retro.puro vector (mock) or a pSUPER.retro.puro vector containing SHP2 shRNA for 48 h (A) or cotransfected with a wild-type form of SHP2 (WT-SHP2) together with a pSUPER. retro.puro vector containing ACSL4 shRNA for 48 h (B and C). MA-10-transfected cells were incubated in serum-free medium with (A) or without AA (B and C) in the presence or absence of 0.5 mM 8Br-cAMP for 1 h. A and B: Steroid production was evaluated by determining progesterone concentrations in the medium. Data are shown as progesterone concentration in nanograms per milliliter. The results are means ± SEM of three (A) or six (B) independent experiments. C: Cells were collected, and the cell lysate proteins were used to analyze ACSL4 expression by Western blot with specific antibodies. Representative immunoblots are shown, and the approximate molecular mass of each immunoblotted protein is indicated. The experiment was performed three times with similar results. The integrated optical density of each band was quantitated. ACSL4 values were normalized against the corresponding β-tubulin band and expressed as arbitrary units (AU). The results are means ± SEM of three independent experiments (C). A: $^{a}P < 0.001$ versus mock + cAMP; $^{b}P < 0.05$ versus SHP2 shRNA + cAMP. B: $^{a}P < 0.05$ versus plasmids + cAMP; $^{b}P < 0.01$ versus plasmids + cAMP; $^{c}P < 0.01$ versus WT-SHP2 + cAMP. C: $^{a}P < 0.001$ versus plasmids + cAMP; $^{b}P < 0.05$ versus plasmids + cAMP; $^{c}P < 0.01$ versus WT-SHP2 + cAMP.

However, we cannot rule out that SHP2 is exerting its actions via alternative mechanisms.

Here we further demonstrated that SHP2 is activated through a cAMP-dependent pathway (24), evidenced by the increase in SHP2 activity after cAMP action in overexpression experiments. Our results are in agreement with the only report published on the subject. Rocchi et al. (24) described that SHP2 is phosphorylated in vitro by PKA and in vivo by ACTH in bovine adrenal cells. In both cases, phosphorylation increases SHP2 activity. Note that this previous report did not describe any physiological function of SHP2 nor did it evaluate the relationship of this modification with the function. Thus, our report is the first to show a relationship between cAMP-dependent activation of SHP2 and its functional role. We cannot state whether the activation is by direct or indirect PKA-dependent phosphorylation of SHP2.

To our knowledge, this is also the first report relating SHP2 to an arachidonic acid-metabolizing enzyme such as ACSL4. Although it is known that ACSL4 induction is regulated by a cAMP/PKA-dependent pathway (11) and by AA (47), we still do not know the mechanism by which SHP2 regulates ACSL4 induction in our system. It is possible to state that ACSL4 expression is regulated at transcriptional and translational levels, depending on the length of cAMP action. At periods shorter than 1 h of cAMP action, ACSL4 protein levels are increased (11), and there is no action on mRNA levels (data not shown), indicating a regulation at the translational level. After 1 h of cAMP action, both protein (Fig. 3C, D) and mRNA (Fig. 3B) levels are increased, indicating that there is additional regulation at the transcriptional level.

The involvement of SHP2 in AA metabolism may explain its role in cell functions. SHP2 has been related to cell proliferation, survival, migration, and differentiation (52, 53). With regard to the mammalian enzyme, stimulatory mutations of the human gene have been found responsible for several abnormalities, such as the pathological activity of Helicobacter pylori (54), Noonan syndrome (33), leukemogenesis (55), and human breast cancer (56, 57). Although the relationship between SHP2 and hormonal imbalance has been analyzed, particularly in diabetes (58–60), the role of SHP2 in these disorders is not yet fully described or understood. Here we report a new physiological role of SHP2 in hormone action, particularly in the hormonal activation of steroidogenesis.

Our identification of SHP2 as an endogenous regulator for ACSL4 expression puts one more piece of the puzzle in place. It helps to identify the PTP involved and describes a regulatory pathway of activation of a fatty acid-metabolizing

enzyme such as ACSL4. Additionally, it provides an attractive target for the treatment of dysregulated cell growth. Studying the mechanism by which SHP2 exerts its action on ACSL4 induction remains a major challenge for future research.

The authors acknowledge Dr. Ben Neel for providing the WT- and CS- SHP2 pRC/CMV to Addgene. Thanks are also due to Carlos F Méndez and Cristina Paz for their critical reading of the manuscript.

REFERENCES

1. Lewin, T. M., J. H. Kim, D. A. Granger, J. E. Vance, and R. A. Coleman. 2001. Acyl-CoA synthetase isoforms 1, 4, and 5 are present in different subcellular membranes in rat liver and can be inhibited independently. *J. Biol. Chem.* **276:** 24674–24679.
2. Van Horn, C. G., J. M. Caviglia, L. O. Li, S. Wang, D. A. Granger, and R. A. Coleman. 2005. Characterization of recombinant long-chain rat acyl-CoA synthetase isoforms 3 and 6: identification of a novel variant of isoform 6. *Biochemistry.* **44:** 1635–1642.
3. Kim, J. H., T. M. Lewin, and R. A. Coleman. 2001. Expression and characterization of recombinant rat acyl-CoA synthetases 1, 4, and 5. Selective inhibition by triacsin C and thiazolidinediones. *J. Biol. Chem.* **276:** 24667–24673.
4. Mashek, D. G., K. E. Bornfeldt, R. A. Coleman, J. Berger, D. A. Bernlohr, P. Black, C. C. DiRusso, S. A. Farber, W. Guo, N. Hashimoto, et al. 2004. Revised nomenclature for the mammalian long-chain acyl-CoA synthetase gene family. *J. Lipid Res.* **45:** 1958–1961.
5. Soupene, E., and F. A. Kuypers. 2008. Mammalian long-chain acyl-CoA synthetases. *Exp. Biol. Med. (Maywood).* **233:** 507–521.
6. Mashek, D. G., L. O. Li, and R. A. Coleman. 2006. Rat long-chain acyl-CoA synthetase mRNA, protein, and activity vary in tissue distribution and in response to diet. *J. Lipid Res.* **47:** 2004–2010.
7. Kang, M. J., T. Fujino, H. Sasano, H. Minekura, N. Yabuki, H. Nagura, H. Iijima, and T. T. Yamamoto. 1997. A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis. *Proc. Natl. Acad. Sci. USA.* **94:** 2880–2884.
8. Sung, Y. K., M. K. Park, S. H. Hong, S. Y. Hwang, M. H. Kwack, J. C. Kim, and M. K. Kim. 2007. Regulation of cell growth by fatty acid-CoA ligase 4 in human hepatocellular carcinoma cells. *Exp. Mol. Med.* **39:** 477–482.
9. Cao, Y., K. B. Dave, T. P. Doan, and S. M. Prescott. 2001. Fatty acid CoA ligase 4 is up-regulated in colon adenocarcinoma. *Cancer Res.* **61:** 8429–8434.
10. Monaco, M. E., C. J. Creighton, P. Lee, X. Zou, M. K. Topham, and D. M. Stafforini. 2010. Expression of long-chain fatty acyl-CoA synthetase 4 in breast and prostate cancers is associated with sex steroid hormone receptor negativity. *Transl. Oncol.* **3:** 91–98.
11. Cornejo Maciel, F., P. Maloberti, I. Neuman, F. Cano, R. Castilla, F. Castillo, C. Paz, and E. J. Podesta. 2005. An arachidonic acid-preferring acyl-CoA synthetase is a hormone-dependent and obligatory protein in the signal transduction pathway of steroidogenic hormones. *J. Mol. Endocrinol.* **34:** 655–666.
12. Maloberti, P., R. Castilla, F. Castillo, F. C. Maciel, C. F. Mendez, C. Paz, and E. J. Podesta. 2005. Silencing the expression of mitochondrial acyl-CoA thioesterase I and acyl-CoA synthetase 4 inhibits hormone-induced steroidogenesis. *FEBS J.* **272:** 1804–1814.
13. Maloberti, P., R. C. Lozano, P. G. Mele, F. Cano, C. Colonna, C. F. Mendez, C. Paz, and E. J. Podesta. 2002. Concerted regulation of free arachidonic acid and hormone-induced steroid synthesis by acyl-CoA thioesterases and acyl-CoA synthetases in adrenal cells. *Eur. J. Biochem.* **269:** 5599–5607.
14. Duarte, A., A. F. Castillo, R. Castilla, P. Maloberti, C. Paz, E. J. Podesta, and F. Cornejo Maciel. 2007. An arachidonic acid generation/export system involved in the regulation of cholesterol transport in mitochondria of steroidogenic cells. *FEBS Lett.* **581:** 4023–4028.
15. Golej, D. L., B. Askari, F. Kramer, S. Barnhart, A. Vivekanandan-Giri, S. Pennathur, and K. E. Bornfeldt. 2011. Long-chain acyl-CoA synthetase 4 modulates prostaglandin E2 release from human arterial smooth muscle cells. *J. Lipid Res.* **52:** 782–793.
16. Sung, Y. K., S. Y. Hwang, M. K. Park, H. I. Bae, W. H. Kim, J. C. Kim, and M. Kim. 2003. Fatty acid-CoA ligase 4 is overexpressed in human hepatocellular carcinoma. *Cancer Sci.* **94:** 421–424.
17. Maloberti, P. M., A. B. Duarte, U. D. Orlando, M. E. Pasqualini, A. R. Solano, C. Lopez-Otin, and E. J. Podesta. 2010. Functional interaction between acyl-CoA synthetase 4, lipooxygenases and cyclooxygenase-2 in the aggressive phenotype of breast cancer cells. *PLoS ONE.* **5:** e15540.
18. Cano, F., C. Poderoso, F. Cornejo Maciel, R. Castilla, P. Maloberti, F. Castillo, I. Neuman, C. Paz, and E. J. Podesta. 2006. Protein tyrosine phosphatases regulate arachidonic acid release, StAR induction and steroidogenesis acting on a hormone-dependent arachidonic acid-preferring acyl-CoA synthetase. *J. Steroid Biochem. Mol. Biol.* **99:** 197–202.
19. Paz, C., F. Cornejo Maciel, C. Mendez, and E. J. Podesta. 1999. Corticotropin increases protein tyrosine phosphatase activity by a cAMP-dependent mechanism in rat adrenal gland. *Eur. J. Biochem.* **265:** 911–918.
20. Poderoso, C., F. C. Maciel, A. Gorostizaga, P. Bey, C. Paz, and E. J. Podesta. 2002. The obligatory action of protein tyrosine phosphatases in ACTH-stimulated steroidogenesis is exerted at the level of StAR protein. *Endocr. Res.* **28:** 413–417.
21. Maciel, F. C., C. Poderoso, A. Gorostizaga, C. Paz, and E. J. Podesta. 2001. LH/chorionic gonadotropin signaling pathway involves protein tyrosine phosphatase activity downstream of protein kinase A activation: evidence of an obligatory step in steroid production by Leydig cells. *J. Endocrinol.* **170:** 403–411.
22. Paz, C., F. Cornejo Maciel, P. Maloberti, L. P. Walsh, D. M. Stocco, and E. J. Podesta. 2002. Protein tyrosine phosphatases are involved in LH/chorionic gonadotropin and 8Br-cAMP regulation of steroidogenesis and StAR protein levels in MA-10 Leydig cells. *J. Endocrinol.* **175:** 793–801.
23. Vilgrain, I., A. Chinn, I. Gaillard, E. M. Chambaz, and J. J. Feige. 1998. Hormonal regulation of focal adhesions in bovine adrenocortical cells: induction of paxillin dephosphorylation by adrenocorticotropic hormone. *Biochem. J.* **332:** 533–540.
24. Rocchi, S., I. Gaillard, E. van Obberghen, E. M. Chambaz, and I. Vilgrain. 2000. Adrenocorticotrophic hormone stimulates phosphotyrosine phosphatase SHP2 in bovine adrenocortical cells: phosphorylation and activation by cAMP-dependent protein kinase. *Biochem. J.* **352:** 483–490.
25. Feng, G. S., C. C. Hui, and T. Pawson. 1993. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science.* **259:** 1607–1611.
26. Vogel, W., R. Lammers, J. Huang, and A. Ullrich. 1993. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science.* **259:** 1611–1614.
27. Dance, M., A. Montagner, J-P. Salles, A. Yart, and P. Raynal. 2008. The molecular functions of Shp2 in the Ras/mitogen-activated protein kinase (ERK1/2) pathway. *Cell. Signal.* **20:** 453–459.
28. Matozaki, T., Y. Murata, Y. Saito, H. Okazawa, and H. Ohnishi. 2009. Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation. *Cancer Sci.* **100:** 1786–1793.
29. Zhou, X., J. Coad, B. Ducatman, and Y. M. Agazie. 2008. SHP2 is up-regulated in breast cancer cells and in infiltrating ductal carcinoma of the breast, implying its involvement in breast oncogenesis. *Histopathology.* **53:** 389–402.
30. Zhou, X. D., and Y. M. Agazie. 2008. Inhibition of SHP2 leads to mesenchymal to epithelial transition in breast cancer cells. *Cell Death Differ.* **15:** 988–996.
31. Ascoli, M. 1981. Characterization of several clonal lines of cultured ley dig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology.* **108:** 88–95.
32. Catt, K. J., T. Tsuruhara, C. Mendelson, J. M. Ketelslegers, and M. L. Dufau. 1974. Gonadotroping binding and activation of the interstitial cells of the testis. *Curr. Top. Mol. Endocrinol.* **1:** 1–30.
33. Gauthier, A. S., O. Furstoss, T. Araki, R. Chan, B. G. Neel, D. R. Kaplan, and F. D. Miller. 2007. Control of CNS cell-fate decisions by SHP-2 and its dysregulation in Noonan syndrome. *Neuron.* **54:** 245–262.
34. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
35. Burridge, K., and A. Nelson. 1995. An in-gel assay for protein tyrosine phosphatase activity: detection of widespread distribution in cells and tissues. *Anal. Biochem.* **232:** 56–64.

36. Markova, B., P. Gulati, P. A. Herrlich, and F. D. Bohmer. 2005. Investigation of protein-tyrosine phosphatases by in-gel assays. *Methods.* **35:** 22–27.
37. Castillo, F., F. Cano, P. Maloberti, R. Castilla, I. Neuman, C. Poderoso, C. Paz, E. J. Podesta, and F. Cornejo Maciel. 2004. Tyrosine phosphates act on steroidogenesis through the activation of arachidonic acid release. *Endocr. Res.* **30:** 623–627.
38. Chan, Y. L., A. Lin, J. McNally, D. Peleg, O. Meyuhas, and I. G. Wool. 1987. The primary structure of rat ribosomal protein L19. A determination from the sequence of nucleotides in a cDNA and from the sequence of amino acids in the protein. *J. Biol. Chem.* **262:** 1111–1115.
39. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65:** 55–63.
40. Alvarez-Díaz, S., N. Valle, J. M. García, C. Peña, J. M. P. Freije, V. Quesada, A. Astudillo, F. Bonilla, C. López-Otín, and A. Muñoz. 2009. Cystatin D is a candidate tumor suppressor gene induced by vitamin D in human colon cancer cells. *J. Clin. Invest.* **119:** 2343–2358.
41. Solano, A. R., L. Dada, and E. J. Podesta. 1988. Lipoxygenase products as common intermediates in cyclic AMP-dependent and -independent adrenal steroidogenesis in rats. *J. Mol. Endocrinol.* **1:** 147–154.
42. Castillo, A. F., F. C. Maciel, R. Castilla, A. Duarte, P. Maloberti, C. Paz, and E. J. Podesta. 2006. cAMP increases mitochondrial cholesterol transport through the induction of arachidonic acid release inside this organelle in Leydig cells. *FEBS J.* **273:** 5011–5021.
43. Sakuma, S., Y. Fujimoto, A. Kitao, H. Sakamoto, H. Nishida, and T. Fujita. 1999. Simultaneous measurement of prostaglandin and arachidonoyl CoA formed from arachidonic acid in rabbit kidney medulla microsomes: the roles of Zn2+ and Cu2+ as modulators of formation of the two products. *Prostaglandins Leukot. Essent. Fatty Acids.* **61:** 105–112.
44. Chen, L., S-S. Sung, M. L. R. Yip, H. R. Lawrence, Y. Ren, W. C. Guida, S. M. Sebti, N. J. Lawrence, and J. Wu. 2006. Discovery of a novel Shp2 protein tyrosine phosphatase inhibitor. *Mol. Pharmacol.* **70:** 562–570.
45. Lewin, T. M., C. G. Van Horn, S. K. Krisans, and R. A. Coleman. 2002. Rat liver acyl-CoA synthetase 4 is a peripheral-membrane protein located in two distinct subcellular organelles, peroxisomes, and mitochondrial-associated membrane. *Arch. Biochem. Biophys.* **404:** 263–270.
46. Cao, Y., K. J. Murphy, T. M. McIntyre, G. A. Zimmerman, and S. M. Prescott. 2000. Expression of fatty acid-CoA ligase 4 during development and in brain. *FEBS Lett.* **467:** 263–267.
47. Cho, Y-Y., M-J. Kang, S. Ogawa, Y. Yamashita, T. Fujino, and T. T. Yamamoto. 2000. Regulation by adrenocorticotropic hormone and arachidonate of the expression of acyl-CoA synthetase 4, an arachidonate-preferring enzyme expressed in steroidogenic tissues. *Biochem. Biophys. Res. Commun.* **274:** 741–745.
48. Flint, A. J., T. Tiganis, D. Barford, and N. K. Tonks. 1997. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. USA.* **94:** 1680–1685.
49. Podesta, E. J., M. L. Dufau, and K. J. Catt. 1976. Adenosine 3′,5′-monophosphate-dependent protein kinase of Leydig cells: in vitro activation and relationship to gonadotropin action upon cyclic AMP and steroidogenesis. *FEBS Lett.* **70:** 212–216.
50. Rae, P. A., N. S. Gutmann, J. Tsao, and B. P. Schimmer. 1979. Mutations in cyclic AMP-dependent protein kinase and corticotropin (ACTH)-sensitive adenylate cyclase affect adrenal steroidogenesis. *Proc. Natl. Acad. Sci. USA.* **76:** 1896–1900.
51. Arakane, F., S. R. King, Y. Du, C. B. Kallen, L. P. Walsh, H. Watari, D. M. Stocco, and J. F. Strauss 3rd. 1997. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *J. Biol. Chem.* **272:** 32656–32662.
52. Mañes, S., E. Mira, C. Gomez-Mouton, Z. J. Zhao, R. A. Lacalle, and C. Martinez-A. 1999. Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Mol. Cell. Biol.* **19:** 3125–3135.
53. Rivard, N., F. R. McKenzie, J. M. Brondello, and J. Pouyssegur. 1995. The phosphotyrosine phosphatase PTP1D, but not PTP1C, is an essential mediator of fibroblast proliferation induced by tyrosine kinase and G protein-coupled receptors. *J. Biol. Chem.* **270:** 11017–11024.
54. Higashi, H., A. Nakaya, R. Tsutsumi, K. Yokoyama, Y. Fujii, S. Ishikawa, M. Higuchi, A. Takahashi, Y. Kurashima, Y. Teishikata, et al. 2004. Helicobacter pylori CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J. Biol. Chem.* **279:** 17205–17216.
55. Xu, R., Y. Yu, S. Zheng, X. Zhao, Q. Dong, Z. He, Y. Liang, Q. Lu, Y. Fang, X. Gan, X. Xu, S. Zhang, Q. Dong, X. Zhang, and G.-S. Feng. 2005. Overexpression of Shp2 tyrosine phosphatase is implicated in leukemogenesis in adult human leukemia. *Blood.* **106:** 3142–3149.
56. Daly, R. J., H. Gu, J. Parmar, S. Malaney, R. J. Lyons, R. Kairouz, D. R. Head, S. M. Henshall, B. G. Neel, and R. L. Sutherland. 2002. OThe docking protein Gab2 is overexpressed and estrogen regulated in human breast cancer. *Oncogene.* **21:** 5175–5181.
57. Martinelli, S., C. Carta, E. Flex, F. Binni, E. L. Cordisco, S. Moretti, E. Puxeddu, M. Tonacchera, A. Pinchera, H. P. McDowell, C. Dominici, A. Rosolen, C. Di Rocco, R. Riccardi, P. Celli, M. Picardo, M. Genuardi, P. Grammatico, M. Sorcini, and M. Tartaglia. 2006. Activating PTPN11 mutations play a minor role in pediatric and adult solid tumors. *Cancer Genet. Cytogenet.* **166:** 124–129.
58. Arrandale, J. M., A. Gore-Willse, S. Rocks, J.-M. Ren, J. Zhu, A. Davis, J. N. Livingston, and D. U. Rabin. 1996. Insulin signaling in mice expressing reduced levels of Syp. *J. Biol. Chem.* **271:** 21353–21358.
59. Krajewska, M., S. Banares, E. E. Zhang, X. Huang, M. Scadeng, U. S. Jhala, G.-S. Feng, and S. Krajewski. 2008. Development of diabesity in mice with neuronal deletion of Shp2 tyrosine phosphatase. *Am. J. Pathol.* **172:** 1312–1324.
60. Asante-Appiah, E., and B. P. Kennedy. 2003. Protein tyrosine phosphatases: the quest for negative regulators of insulin action. *Am. J. Physiol. Endocrinol. Metab.* **284:** E663–670.