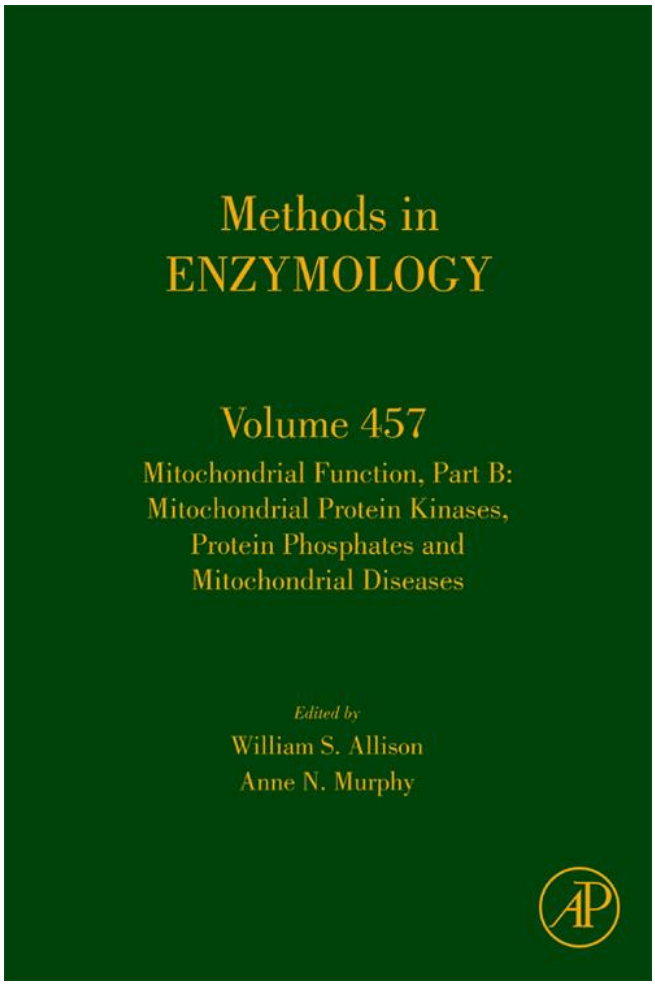

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CHAPTER TEN

Detection of a Mitochondrial Kinase Complex That Mediates PKA–MEK–ERK-Dependent Phosphorylation of Mitochondrial Proteins Involved in the Regulation of Steroid Biosynthesis

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Abstract

In order to achieve the goal of this article, as an example we will describe the strategies followed to analyze the presence of the multi-kinase complex at the mitochondria and the posttranslational modification of two key mitochondrial proteins, which participate in the regulation of cholesterol transport across the mitochondrial membranes and in the regulation of steroid biosynthesis. Hormones, ions or growth factors modulate steroid biosynthesis by the posttranslational phosphorylation of proteins. The question still remains on how phosphorylation events transmit a specific signal to its mitochondrial site of action.

Cholesterol transport requires specific interactions in mitochondria between several proteins including a multi-kinase complex. The presence of this multi-kinase complex at the mitochondria reveals the importance of the posttranslational modification of mitochondrial proteins for its activity and functions. The activation of PKA triggers the posttranslational modification of the mitochondrial acyl-CoA thioesterase (Acot2), which releases arachidonic acid (AA) in the mitochondria, and the activation of a kinase cascade that leads to the phoshorylation of the steroidogenic acute regulatory (StAR) protein. The function of StAR is to facilitate the access of cholesterol to the first enzyme of the biosynthesis process and its induction is dependent on Acot2 and intramitochondrial AA release. Truncation of the StAR protein is associated with the steroid deficiency disease, congenital lipoid adrenal hyperplasia.

1. Introduction

The number of mitochondria present in a cell corresponds closely to the functional status or to the energy requirements of a given tissue. For example, the heart, which contracts constantly, has the highest mitochondrial content of all tissues, whereas type Hx muscle fibers, which are recruited only periodically, have a low mitochondrial volume per fiber. Although the primary role of mitochondria is ATP provision, these organelles are also involved in other processes that are important for cellular function. These include calcium homeostasis, intracellular signal transduction and regulation of apoptosis. For instance, steroid biosynthesis is a complex processes that proceeds after a first step of enzymatic conversion of cholesterol that occurs in the mitochondria once cholesterol is transported across mitochondrial membranes. Thus, steroidogenic cells *contain* a large amount of mitochondria, part of which is dedicated to steroid production.

We describe here the strategies used to analyze the presence of a multi-kinase complex at mitochondria and the posttranslational modifications of two key mitochondrial proteins that participate in the regulation of

cholesterol transport across the mitochondrial membranes and the regulation of steroid biosynthesis. In all steroidogenic tissues (testis, adrenal gland, ovary, placenta, and brain), the acute stimulation of steroid synthesis is under the control of a number of phosphorylation-dependent events that implicate the activation of numerous protein kinases. The cyclic-AMP (cAMP)-dependent protein kinase (PKA), the protein kinase C (PKC), the calcium/calmodulin-dependent protein kinase (CamK) and the mitogen activated protein kinases (MAPKs) are among the kinases involved in steroidogenesis. Thus, hormones, ions or growth factors modulate steroid biosynthesis by posttranslational phosphorylation of proteins. The question still remains *on* how phosphorylation events relay a specific signal to its mitochondrial site of action.

Cholesterol transport requires specific interactions in mitochondria between several proteins including the voltage-dependent anion channel (VDAC), the peripheral benzodiazepine receptor (PBR, currently named translocator protein or TSPO), the PBR/TSPO-associated protein (PAP7), an acyl-CoA-thioesterase (Acot2), PKA, the extracellular signal-regulated kinase (ERK), its upstream activator (MEK), and the steroidogenic acute regulatory (StAR) protein. The presence of a multi-kinase complex *in mitochondria* shows the importance of the posttranslational modification of mitochondrial proteins for its activity and functions. One of the mitochondrial proteins phosphorylated throughout the activation of the kinase cascade is StAR. The function of StAR is to facilitate the access of cholesterol to the first enzymatic step of the biosynthetic process. Truncation of StAR protein associates with a human steroid deficiency called congenital lipoid adrenal hyperplasia (Lin *et al.*, 1995). The second protein phosphorylated through a PKA-dependent pathway is the mitochondrial acyl-CoA thioesterase Acot2, which releases arachidonic acid (AA) in the mitochondria, and it is essential for StAR induction (Maloberti *et al.*, 2002, 2005).

2. Steroidogenic Cells

Steroid biosynthesis is a process finely regulated by hormones, ions, and growth factors that trigger a number of signal transduction pathways involving the activation of different kinases and phosphatases. Thus, steroidogenic cells constitute an appropriate system for the study of protein phosphorylation. Apart from animal models, cell lines are also available as study material. Some of the most used and accepted cell lines are MA-10, Y1, H295R, mLTC and a series of modifications. For example, Y1 cells were used to generate mutants defective in PKA or adenylylcyclase activity, Y1(kin) and Y1(cyc) cells, respectively (Al-Hakim *et al.*, 2004; Rae *et al.*, 1979; Schimmer *et al.*, 1977). There are several advantages in using cell lines.

First, the model is based upon a unique type of cells, thus facilitating the interpretation of results. Second, a large amount of material is easily and rapidly obtained. Third and most important, steroid production is easily evaluated in cell culture media by radioimmunoassay. All of our studies were performed in adrenocortical or interstitial testicular primary cell cultures or using MA-10 Leydig or Y1 adrenocortical cells.

The MA-10 cell line is a clonal strain of mouse Leydig tumor cells that produce progesterone rather than testosterone as the major steroid (Ascoli, 1981). The growth medium consists of Waymouth MB752/1, 20 m*M* HEPES, 50 μg/ml gentamycin and 15% horse serum. Cells are grown in a humidified atmosphere at 36.5 °C containing 5% CO_2. Stimulation of the cells is performed with chorionic gonadotropin (CG) or 8Br-cAMP in serum-free Waymouth medium, and steroid production is evaluated by determination of progesterone concentrations in the incubation media.

3. Analysis of a Mitochondrial Kinase Complex

3.1. Involvement of PKA, MEK1/2, and ERK1/2 in mitochondrial function

Adrenocorticotropin (ACTH) and Luteinizing Hormone (LH) stimulate steroid production in adrenocortical and testicular Leydig cells, respectively, through a mechanism that involves the activation of PKA. One of the effectors of PKA in those systems is StAR protein. Murine StAR is phosphorylated by PKA in Ser194 while Ser197 is the phosphorylated residue in human StAR, a posttranslational modification that regulates its cholesterol transport capacity.

A-kinase anchor proteins (AKAPs) enhance cAMP-dependent pathways by anchoring PKA near its cellular substrates (Feliciello *et al.*, 2001; Rubin, 1994). It has been demonstrated that murine AKAP121 tethers PKA to the mitochondrial outer surface (Angelo and Rubin, 2000; Diviani and Scott, 2001). In view of this finding, and taking into account the mitochondrial localization of StAR, it seems possible that AKAPs could provide spatial integration between PKA and StAR.

Other kinases involved in the activation of steroid production are ERK1/2 and its upstream activator MEK1/2 which act through genomic and non-genomic effects. The site of action of MEK1/2–ERK1/2 is located between PKA activation and cholesterol transport. Evidence for this type of regulation was obtained by studying steroid production in cells incubated with inhibitors of MEK activation such as U0126 or PD98095. The activation of the MEK1/2–ERK1/2 cascade is dependent on PKA activation, as demonstrated by siRNA experiments described below. The role of ERK in the regulation of steroid biosynthesis was also confirmed by studies

using an overexpression approach of wild-type ERK1 and different mutated forms of the kinase (also described below).

3.1.1. Transient transfection of siRNA for PKA using cationic lipids reagents

Small interference RNA is one of the most important research tools for gene silencing since it is target-specific and the knock down efficiency of the specific protein can be easily assessed by immunoblot using antibodies against the targeted protein. The gene silencing strategy is of higher specificity than that achieved with inhibitors such as H89 or PKI commonly used in the study of PKA. Additionally, the siRNA may be directed to different isozymes of PKA making it possible to study the effect of a particular isoform within a system.

Transfection with cationic lipid reagents is considered the most appropriate and less harmful procedure for the introduction of siRNAs into cells. Our siRNA studies were carried out using MA-10 cells. The lipofection mixture consisted of 100 n*M* of siRNA of the α isoform of the catalytic subunit of PKA and Lipofectamine 2000 Reagent in Opti-MEM medium (Invitrogen) with 15% horse serum and without antibiotics according to Maloberti *et al.* (2005). Cells were transfected for approximately 48 h by adding the mixture to Waymouth medium without antibiotics with 15% horse serum. Transfection efficiency was monitored by transfection of the cells with a plasmid containing the coding sequence of the enhanced green fluorescent protein (pRc/CMVi-EGFP). Following transfection, the medium was replaced with complete Waymouth and cells were allowed to grow for additional 12 h, after which horse serum was removed and the culture remained serum-deprived for an extra 24 h period. ERK1/2 activity was evaluated in the mitochondrial fraction of MA-10 cells (obtained as described below) by means of an enzyme-linked immunosorbent assay (ELISA) that detects the phosphorylated form of the kinase (Sigma, St. Louis, MO) following stimulation of the cells with 0.5 m*M* 8Br-cAMP. Full activation of ERK1/2 in stimulated MA-10 cells was strictly dependent on the activation of α isoform of the catalytic subunit of PKA in mitochondria.

3.1.2. Constructs of wild-type ERK and ERK H230R for transfection and expression in MA-10 cells

Although inhibitors of MEK1/2 activity are commonly used during functional studies, these compounds are not entirely selective and can affect other kinases. An alternative approach to study the participation of ERK1/2 in a given pathway is the use of molecular biology techniques that modify the levels of the protein in question. That procedure allows for the overexpression of wild-type ERK 1/2 or of negative dominants of the kinase. In our case, we overexpressed wild-type ERK 1/2 and an inactive

form of ERK2, the H230R variant, which fails to interact with MEK1 but retains the ability to interact with MEK2 in a weakened fashion (Robinson *et al.*, 2002) in MA-10 cells.

3.1.3. Transient transfection of ERK and ERK H230R by electroporation

MA-10 cell cultures growing in the logarithmic phase are harvested by trypsin digestion, centrifuged and resuspended at a final concentration of 1.6×10^7 cells/ml. Approximately 8×10^6 cells were placed in an electroporation cuvette in 400 μl of antibiotic-free Waymouth MB752/1 medium containing 15% horse serum in the presence of 30 μg of a midipreparation of empty 3xflag-CMV7 plasmid as mock control, 3xflag-CMV7-ERK2 wild type or 3xflag-CMV7-ERK2 H230R mutated form of ERK2. The cell suspension was gently mixed and electropored with a pulse of 0.3 V and 950 μF in the Gene Pulser® II system (Bio-Rad). After the pulse, the cuvettes containing the transfected cells were kept on ice for 10 min and then seeded in 75 cm^2 flasks. After 12 h, the transfection medium was replaced with complete medium and allowed to grow for additional 36 h before the experiment. Again, evaluation of transfection efficiency was monitored by means of a plasmid containing the cDNA for EGFP (pRc/CMVi-EGFP). Usually, 30–35% transfection efficiency was achieved using the described electroporation protocol.

Overexpression of a wild-type form of ERK2 in MA-10 cells resulted in an increment of 8Br-cAMP-stimulated steroid production, while the inactive form of ERK2, H230R variant, failed to stimulate steroidogenesis (Poderoso *et al.*, 2008), thus confirming the role of active ERK1/2 in the regulation of steroid synthesis.

3.2. Localization and activation of MEK and ERK at the mitochondria by western blot and confocal microscopy

The mitochondrion is the key organelle in the steroid production process. Given the proposed role of MEK/ERK in steroidogenesis, it is of interest to explore the possible mitochondrial localization of MEK1/2, ERK1/2, phospho-MEK, and phospho-ERK. For that purpose, we used a combination of western blot and confocal microscopy.

3.2.1. Subcellular fractionation and assessment of the purity of each fraction

For subcellular fractionation, cells were rinsed with PBS, scrapped with 1 ml ice-cold PBS, and collected by centrifugation at 800*g* for 10 min. Cells were then resuspended in MSHE buffer (250 m*M* Mannitol, 219 m*M* Sacarose, 0.02% EGTA, 0.1% BSA, 1.8 m*M* Hepes pH 7.4) containing protease and phosphatase inhibitors (10 μM leupeptine, 2 μg/ml aprotinine, 1 μM

pepstatine, 1 m*M* PMSF, 2 m*M* sodium orthovanadate, 25 m*M* sodium fluoride, and 40 m*M* β-Glycerophosphate) and mechanically disrupted by 60 strokes of a 1 ml syringe. Following homogenization, cells were centrifuged for 10 min at 5000*g* to collect nuclei. The resulting supernatant was centrifuged for 20 min at 15,000*g* to obtain the mitochondrial (pellet) and cytosolic (supernatant) fractions. The mitochondria-enriched fraction was washed once in MSHE buffer and resuspended in the same buffer before use. The nuclear fraction was rinsed once with washing solution (10 m*M* Tris pH 7.4, 1.5 m*M* EDTA, 10% Glycerol, 0.01% NP-40), resuspended in a hyperosmolar solution containing 10 m*M* Tris pH 7.4, 1.5 m*M* EDTA, 10% Glycerol, 0.4 *M* KCl, and homogenized by 15 strokes of a 1 ml syringe. Nuclear soluble proteins were finally obtained by centrifugation for 30 min at 25,000*g*.

Purity of the different subcellular fractions was determined by measurement of the activity of specific enzymes or by immunoblot detection of specific marker proteins.

Lactate dehydrogenase activity (cytosolic index) was assayed spectrophotometrically by following NADH oxidation at 340 nm. Twenty-five micrograms of protein were added to a 1 ml reaction buffer containing 100 m*M* KPi, pH 7.0; 30 m*M* piruvic acid; 1% Triton X-100; and 220 μM NADH. Activity is expressed as micromoles of NADH consumed per milligram of protein per minute.

NADH-cytochrome-c reductase (Complexes I-III) activity (mitochondrial index) was assayed spectrophotometrically by determining cytochrome c reduction at 550 nm in the presence of 30 μM cytochrome c, 1 m*M* potassium cyanide and 150 μM NADH as electron donor. The velocity of the reaction was determined as a pseudo-first-order constant and expressed as *k* per minute per milligram of protein.

For immunoblot control of subcellular fractionation, DNA polymerase II was used as nuclear marker and the 39 kDa subunit of the NADH-cytochrome c reductase (complex I) as mitochondrial marker. Primary antibodies were incubated for 16 h at 4 °C and specific binding was detected by horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence or the advanced ECL detection system (Amersham). For quantitative analysis, densitometry was performed using a Storm Phosphorimager scanner (Amersham Biosciences, Sweden) and band intensities were analyzed using ImageQuant 5.2 software.

Each subcellular fraction was assumed at least 90% pure by enzymatic and western blot determinations.

3.2.2. Western blot analysis of ERK1/2 and MEK1/2

Samples containing equal amounts of proteins from the different subcellular fractions were subjected to SDS–PAGE in 12% polyacrilamide gels. Separated proteins were electrotransferred to PVDF membranes that were incubated with

polyclonal anti-phospho-ERK1/2 or polyclonal anti-phospho-MEK1/2 for the detection of the phosphorylated form of the kinases. Protein loading was evaluated by re-incubation of the membranes with anti-ERK1/2 and anti-MEK1/2, that detect the non-phosphorylated form of the kinases, after striping of the membranes in a stripping buffer containing 62.5 m*M* Tris–HCl pH 6.8; 2% SDS; 100 m*M* β-mercaptoethanol.

3.2.3. Confocal analysis of ERK1/2

MA-10 cells were grown to approximately 60–80% confluence on poly-L-lysine glass coverslips, stimulated and stained with a specific mitochondrial marker, MitoTracker Red 580 (Molecular probes) (300 n*M*, 45 min at 37 °C) following fixation in 4% paraformaldehyde in PBS for 10 min at room temperature and blocking in 1% BSA; 0.3% Triton X-100; PBS, pH 7.4, in a humidified chamber for 1 h. Cells were also incubated overnight at 4 °C with anti-phosphoERK1/2. Cells were further incubated for 1 h at room temperature with a Cy2-conjugated goat anti-rabbit antibody and coverslips mounted onto slides using Fluorsave mounting media (Calbiochem) for confocal laser scanning microscopy using a Nikon C1 microscope 60×/1.40 (IByME, UBA, Argentina), objective Plan-Apo 40×/0.95. The excitation lines are 488 (blue) and 544 nm (green), emission filters are 515–530 nm (green) and 570-LP (red).

Using immunoblot and confocal microscopy, we located pERK1/2 to the mitochondria of MA-10 cells after stimulation with hCG or 8Br-cAMP (Fig. 10.1A). ERK1/2 phosphorylation was rapid, with a marked increase already 5 min after 8Br-cAMP stimulation, and a slow and progressive decrease of the signal during the first hour of 8Br-cAMP action. hCG induced the activation of ERK1/2 activation with a similar kinetic of that produced by 8Br-cAMP.

Confocal microscopy corroborated 8Br-cAMP-induced activation of mitochondrial ERK1/2 (Fig. 10.1B). As observed with immunoblots, pERK1/2 co-localized with mitochondria after 8Br-cAMP action, an effect observable as early as 5 min and still evident 30 min later.

Two different pools of MEK1/2, containing the non-phosphorylated and the phosphorylated forms of the kinase were detected in the cytosol and mitochondria of MA-10 cells. 8Br-cAMP treatment of the cells induced prolonged MEK1/2 phosphorylation in mitochondria, but had a less pronounced effect on the cytosolic form of the kinase.

cAMP-induced increase in mitochondrial pERK1/2 and pMEK1/2 was abolished by treatment of the cells with the PKA inhibitor H89 and by PKA knockdown experiments. Accordingly, mitochondrial PKA activity increased after 5 min of 8Br-cAMP action (data not shown) in parallel with the appearance of the phosphorylated forms of MEK1/2 and ERK1/2 in the organelle.

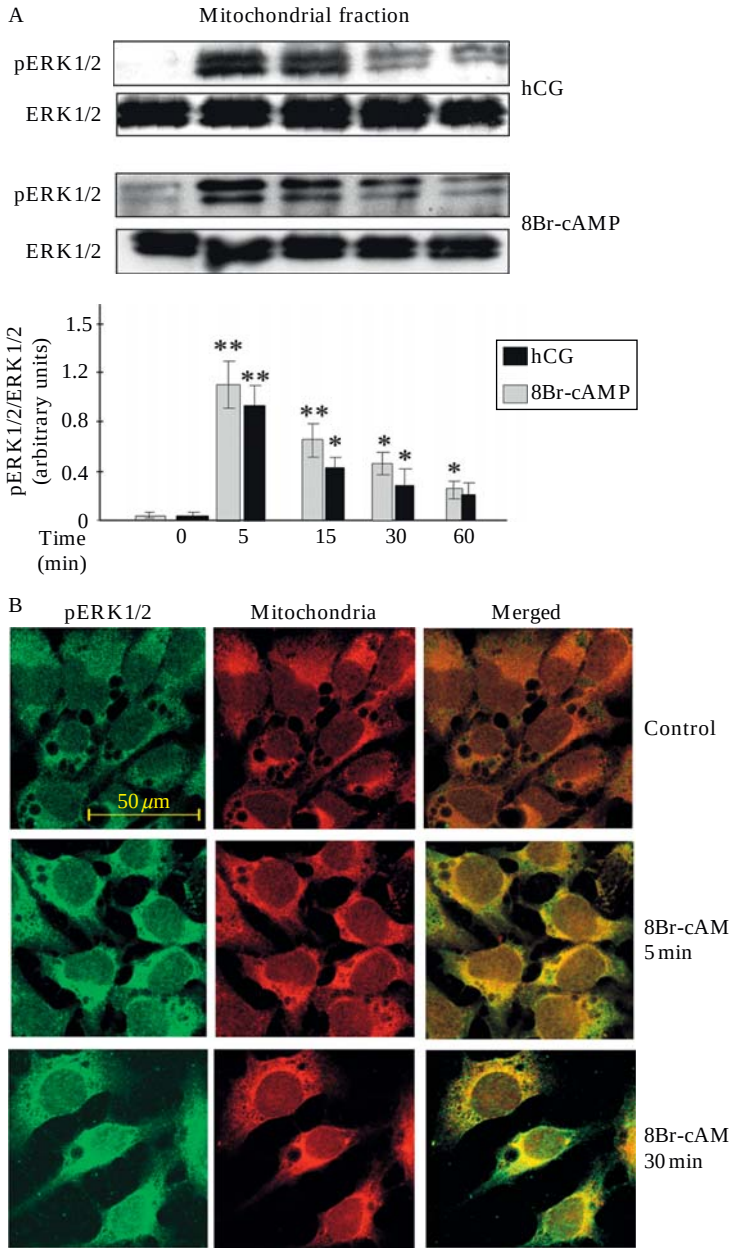

Figure 10.1 Subcellular distribution of hCG/8Br-cAMP-stimulated pERK1/2 activation. MA-10 cells were incubated with or without 20 ng/ml of hCG or 0.5 m*M* 8Br-cAMP (A) for the indicated times. Next, subcellular fractions were obtained and 40 μg of the total protein of each fraction were subjected to SDS–PAGE and Western blot to

3.3. Functional analysis of kinase interaction at the mitochondria

It is important to localize protein kinases in mitochondria, but it is even more important to analyze the action of a given protein on a given mitochondrial function. For this purpose, one approach is to add the purified protein to a fresh preparation of isolated mitochondria and to evaluate the resulting function. In our case, we studied the action of exogenously added ERK1 and PKA to mitochondria enriched in StAR protein and evaluated the effect on steroid production.

3.3.1. StAR construct and MA-10 cell transfection to enrich the mitochondria of non-stimulated cells with StAR

StAR cDNA (accession N° BC082283) is obtained by PCR from MA-10 cells using primers designed according to the published sequence of mouse StAR. Forward (5′-GGACCTTGAAAGGCTCAGGAAGAACAACCC-3′) and reverse (5′-GGATTAGTAGGGAAGTCGGCACAATGATGG-3′) primers amplify a 1440-bp fragment, corresponding to the 37 kDa protein. The amplified sequence is cloned into pGEM-T easy plasmid (Promega), a basic cloning vector. T vectors are a specific type of cloning vectors that get their name from the T overhangs added to a linearized plasmid. These vectors take advantage of the A overhangs on PCR products after amplification with Taq DNA polymerase by providing compatible ends for ligation.

Following ligation, the fragment containing the double-stranded sequence of StAR cDNA was removed from the vector by EcoRI digestion. The digestion product was subjected to agarose gel electrophoresis and the 1.4 kpb StAR cDNA was subcloned into the eukaryotic expression plasmid, pRc/CMVi. The ligation provided both sense and antisense orientations that were used for transformation of XL-1 *E. coli*. Several clones were then screened, and sense and antisense plasmids obtained by midipreparations. The resulting material was transfected, as described above, in MA-10 cells, and mitochondria obtained from the cells were used for studies in a cell-free assay.

detect phospho-ERK1/2 (indicated with pERK1/2). After stripping, total ERK1/2 (indicated with ERK1/2) was detected in the same membrane. The Western blots show the results of a representative experiment performed three times. Graphic bar below shows the quantification of immunoblots above; the intensity of the bands was quantitated using total ERK1/2 as loading control. Bars denote relative levels of pERK1/2 presence in arbitrary units. **$p = 0.01$, *$p = 0.05$ vs. time 0. (B) Immunofluorescent staining for pERK1/2 (green) and mitochondria (red) in MA-10 cells after treatment with or without 0.5 m*M* of 8Br-cAMP for the indicated times. Merged images are shown in the right panel.

3.3.2. Cell-free assay as a tool to evaluate mitochondrial function

To test the effect of active ERK1/2 on cholesterol import and progesterone synthesis, we made use of a cell-free assay that allows the determination of progesterone production by isolated mitochondria. This cell-free assay relies on the ability of isolated mitochondria to respond to exogenously added factors if derived from unstimulated cells. Thus, our mitochondrial source consists of non-stimulated MA-10 cells previously enriched in StAR protein by transfection of the corresponding expression construct.

Isolated mitochondria were then incubated (30 °C for 20 min with gentle agitation) in the absence or presence of 50 μM cholesterol as substrate. To analyze the effect of purified kinases on isolated mitochondria, commercially available ERK1 and PKA were used. Mitochondria were incubated in the presence or absence of 300 ng of human recombinant wild-type active ERK1-GST alone or in combination with active PKA catalytic subunit (1 IU). A mutated inactive form of ERK1 (K71A ERK-GST, 500 ng) was also used as control of ERK1 activity. A single amino acid change in ERK1 generates the K71A mutant that lacks autophosphorylation ability and myelin basic protein phosphotransferase activities. K71A is less efficiently phosphorylated by MEK1/2 than its wild-type counterpart (approximately 80%) and that results in partial activation of the myelin basic protein phosphotransferase activity (20%). After that, mitochondria are pelleted by centrifugation at 15,000*g* for 20 min; media is collected to assess progesterone production by radioimmunoassay. Mitochondrial protein content is analyzed by SDS–PAGE and Western blot to visualize the resulting localization of endogenous and exogenous proteins after the recombination of all these components.

Immunoblot profiles indicate that at the end of the assay, StAR protein is still present in the mitochondria of cells transfected with a sense cDNA plasmid (Fig. 10.2A, western blot of StAR) and that the exogenously added proteins are effectively incorporated and phosphorylated in the organelle (Fig. 10.2A, western blot of PKA catalytic subunit, pERK1/2, and ERK1/2). In this regard, it is worth mentioning that an example of the interaction between the exogenously added proteins and the endogenous mitochondrial protein is the fact that mitochondrial MEK is phosphorylated by the addition of PKA catalytic subunit (Fig. 10.2A, Western blot pMEK1/2). After supplementation of isolated mitochondria with recombinant wild-type ERK1, progesterone yield increased (Fig. 10.2B, bar b vs. a) while addition of the inactive ERK1 mutant (K71A) had no effect on steroid production (Fig. 10.2B, bar c vs. a). The increment in progesterone production was more pronounced when isolated mitochondria were supplemented with the catalytic subunit of recombinant PKA and wild-type ERK1 than when they were challenged with wild-type ERK1 alone (Fig. 10.2B, bar d vs. b). Expectedly, addition of K71A mutant of ERK1 to mitochondria markedly reduced progesterone production (Fig. 10.2B, bar e vs. d).

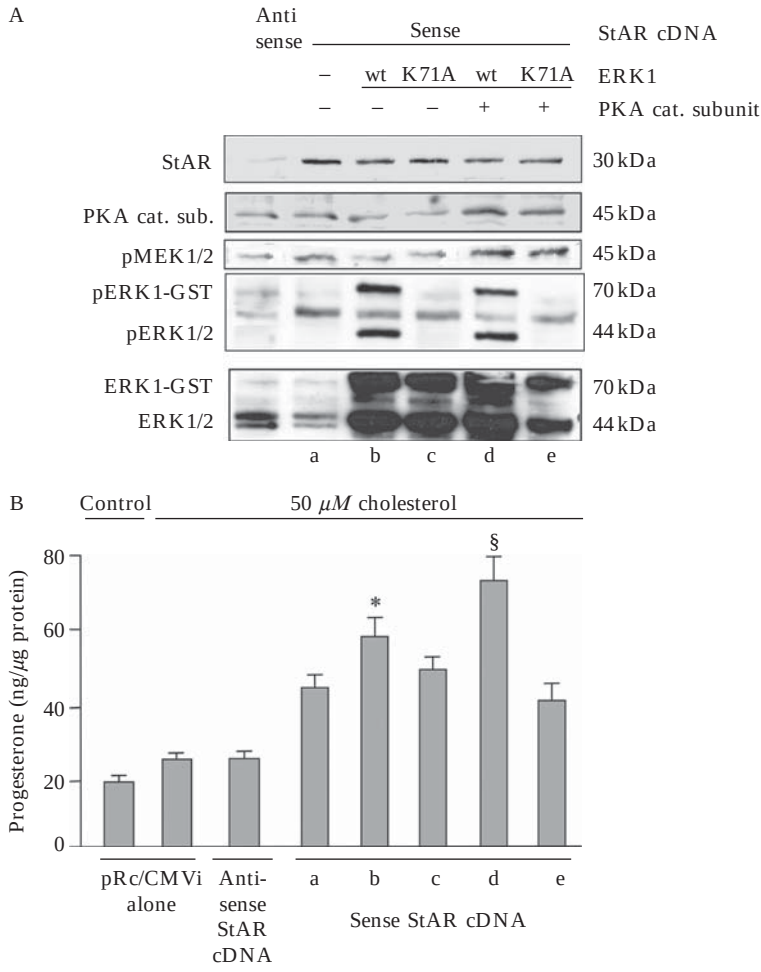

Figure 10.2 PKA and ERK1/2 are strictly required to achieve maximal progesterone production by isolated mitochondria. MA-10 cells were transiently transfected by electroporation with an empty vector (pRc/CMVi) or with a vector containing StAR cDNA (sense or antisense orientations). Mitochondria were incubated in the presence of cholesterol as substrate (a) in the presence of wild-type ERK1-GST protein alone (b) or together with PKA catalytic subunit (d). The mutated inactive form of ERK1-GST (K71A) was also used (c and e). After the indicated incubations, mitochondria were pelleted and subjected to SDS–PAGE and Western blot (A). Specific antibodies that recognize StAR protein, the catalytic subunit of PKA, pMEK1/2, pERK1/2, and total ERK1/2 were used. The panel shows representative Western blots of three independently performed experiments. P4 production is shown in (B). Data are expressed as means ±SD ($n = 3$). $^{\star}p = 0.05$ bar b vs. bar a and $^{\S}p = 0.05$ bar d vs. bar b.

3.4. StAR as substrate of a kinase complex

3.4.1. Sequence analysis

Two important motifs should be present in a protein to be considered as *an* ERK substrate: a docking site for ERK and a phosphorylation consensus site. The typical docking site known as the D domain in ERK substrates shows the sequence **K**T**K**LTWL**LSI** (Fantz *et al.*, 2001) while the phosphorylation consensus site is an SP motif.

A potential docking site in StAR sequence was located between amino acids 235 and 244, with a highly conserved profile between species. Although a search for ERK consensus sites in StAR using the Expasy Prosite database (http://expasy.org/prosite/) resulted negative, a detailed inspection of StAR's sequence indicated two Ser-Pro motifs, Ser232 and Ser277 as putative phosphorylatable residues in murine StAR mature protein. Interspecies analysis of StAR sequence indicated that Ser277 is less conserved than Ser232. In addition, Ser232 (PLAGS^{232}PS) lies adjacent to the docking D domain (-2) in a similar fashion as in the classical ERK1/2 substrate Elk-1 (Bardwell *et al.*, 2003) except for the inverted position with respect to the docking domain. In addition, Elk-1's SP motif contains a proline residue in position-1 or -2, whereas StAR has a conserved proline at position-3. As in other substrates, proline is followed by leucine and there is no acidic residue in the motif.

Our findings regarding ERK's docking and phosphorylation sites in StAR protein emphasizes the need for a careful sequence revision to find putative sites for any kinase. Apart from open access databases, it may be necessary to conduct manual alignments of the protein sequence under study.

3.4.2. Interaction of StAR with the kinase complex: Pull-down assay

One possible approach to the study of protein–protein interactions consists of pull-down assays that are based on the precipitation of a known cellular protein and its possible partner(s). Often, pull-down assays are performed after overexpression of one of the proteins suspected to take part in the interaction to maximize the chances of detection. The overexpressed protein is usually tagged in some way, which simplifies the separation of the recombinant protein itself and any other cellular components that interact with it. Finally, a western blot analysis is performed using antibodies against the proteins under study including a loading control.

For our assays using StAR-enriched mitochondria, we isolated the mitochondrial and cytosolic fractions from cells previously stimulated with 8Br-cAMP for 3 h. Cytosolic or mitochondrial proteins (500 or 300 μg, respectively) were then incubated in the presence of human recombinant ERK1-GST bound to agarose beads (Stressgen) previously activated as described by the manufacturer. The pull-down was performed during

16 h at 4 °C in a buffer containing: 50 m*M* Tris pH 7.4; 150 m*M* NaCl; 1 m*M* EDTA; 1 m*M* EGTA; 10% glycerol; 0.5% Nonidet P-40; 1 m*M* $MgCl_2$; 1 m*M* PMSF; 5 μg/ml leupeptin; 5 μg/ml pepstatin; 5 μg/ml aprotinin; 25 m*M* NaF, and 1m*M* sodium orthovanadate. After incubation, agarose beads were precipitated by low-speed centrifugation, obtaining an agarose-protein pellet which was washed in the same buffer and boiled for 5 min in SDS–PAGE loading buffer. The sample was then subjected to SDS–PAGE and immunoblot analysis with antibodies against StAR and phospho-ERK1/2 as loading control.

Treatment of the two subcellular fractions (cytosol and mitochondria) with pERK1-GST showed that StAR interacts with pERK1 only in the mitochondrial fraction (Fig. 10.3).

3.4.3. Mutation of the putative ERK1/2 phosphorylation site: Obtention of recombinant wild type and S232A mutated StAR protein

cDNA for the mature form of StAR (30 kDa) is obtained by RT-PCR from MA-10 cells with the following primers: the forward 5′-GGATCCG-CAGGGTGGATGGGTCAA- 3′ and the reverse 5′-GGATTAGTAGG-GAAGTCGGCACAATGATGG- 3′ that amplify a 1200-pb fragment. The obtained fragment is then cloned in the pGEM-T easy plasmid, and further subcloned after purification in the pGEX-4T-3 plasmid (Promega) to add a GST tail to the recombinant protein. The pGEX Vector is included in the GST Gene Fusion System with a Taq promoter for chemically inducible, high-level expression in any *E. coli* host. Very mild elution conditions are needed for release of fusion proteins from the affinity matrix,

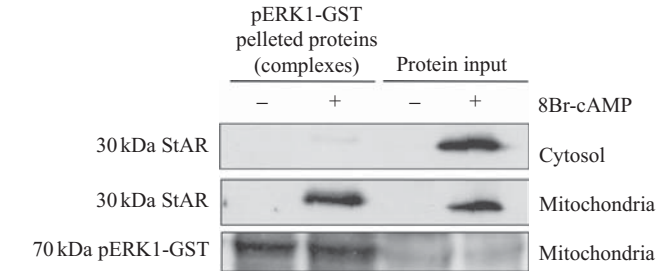

Figure 10.3 StAR interacts with pERK1/2 specifically in mitochondria. MA-10 cells were treated with or without 0.5 m*M* of 8Br-cAMP for 3 h; cytosolic and mitochondrial subcellular fractions were obtained and incubated in the presence or absence of human pERK1-GST bound to agarose beads. Input and pelleted proteins bound to pERK1-GST (complexes) were analyzed by SDS–PAGE and Western blot. The immunoblots show the bands corresponding to StAR and pERK1/2, as loading control. Data are representative of three independently performed experiments.

thus minimizing effects on antigenicity and functional activity of the protein of interest.

StAR cDNA in the sense orientation was subjected to site-directed mutagenesis obtaining a mutant cDNA, StAR S232A, where serine 232 is changed to alanine. Both, wild-type StAR and the S232A mutant were expressed in BL-21 *E. coli*, recombinant proteins purified by glutathione affinity chromatography (GST Purification Module, Amersham Biosciences) and subjected to thrombin proteolytic cleavage. Obtained proteins were used for *in vitro* phosphorylation experiments.

3.4.4. *In vitro* phosphorylation of recombinant wild type and S232A StAR by wild type and inactive ERK1

As stated earlier, *in vitro* phosphorylation assays are appropriate to demonstrate the viability of a phosphorylation of a protein by a given kinase. Variants carrying a mutation that changes a phosphorylatable amino acid for a non-phosphorylatable are widely used to demonstrate the location of the phospho-acceptor amino acid in the substrate.

Thus, to study the phosphorylation of StAR by ERK1/2, we performed an *in vitro* phosphorylation assay using a purified preparation of mature recombinant StAR protein (30 kDa) and its S232 variant as substrates and two forms of ERK1, the wild-type active and an inactive form of the kinase, and [γ-^{32}P]ATP. Approximately 30 μg of recombinant wild type or S232A StAR were used as substrate for the *in vitro* phosphorylation assay. The reaction was performed in 1.5 ml tubes (Eppendorf) in a reaction buffer containing 20 m*M* MOPS pH 7.5; 10 m*M* Mg_2Cl_2; 5 m*M* EGTA, 2×10^{-4}% Tween-20; 10 μCi [^{32}P]γ-ATP; 100 μM ATP; 1m*M* sodium orthovanadate; 1 m*M* DTT). Wild-type StAR was incubated in the presence or absence of 150 ng of human His-tagged ERK1 catalytically active (Calbiochem) or 300 ng of the mutated and inactive form of ERK1 (K71A ERK-GST) or 1 IU of PKA catalytic subunit (Cell Signaling). PKA was included in our phosphorylation assay by two reasons. First, as a control, since it is known that StAR is phosphorylated by PKA and second, to investigate whether PKA is necessary for the subsequent phosphorylation of StAR by ERK. The phosphorylation status of StAR was analyzed in the presence or absence or 10 μM cholesterol. The reaction was performed for 30 min at 30 °C at a final volume of 30 μl and stopped with 10 μl of SDS–PAGE loading buffer (4×), heated at 80 °C for 5 min and subjected to SDS–PAGE. Polyacrylamide gels were stained, dried and then exposed to autoradiography and scanned using a PhosphorImager.

Wild-type StAR was indeed phosphorylated by wild-type ERK1. Expectedly, the inactive mutant of the kinase (K71A) failed to phosphorylate both wild-type StAR and the S232A variant (Fig. 10.4A and B). Interestingly, phosphorylation of StAR by wild-type ERK1 is enhanced in the presence of cholesterol (Fig. 10.4A), while prior phosphorylation of

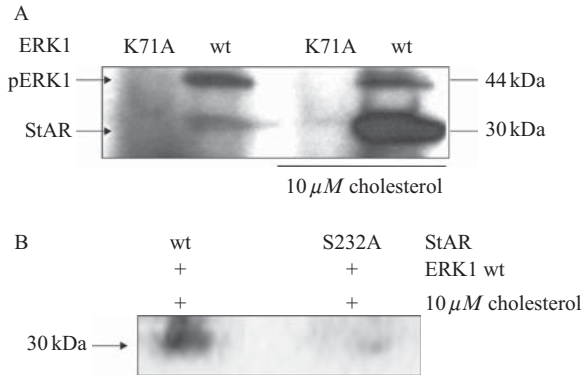

Figure 10.4 **StAR is phosphorylated in Ser232 by ERK1 in a cholesterol-dependent manner. Thirty micrograms of recombinant purified 30 kDa wild-type StAR were incubated in the absence or in the presence of 10 μM of cholesterol (A) together with constitutively active His-tagged wild-type ERK1 (wt) or the mutated inactive form of ERK1 (K71A) (A). After the phosphorylation assays, phosphorylated protein levels were analyzed by SDS–PAGE and autoradiography. (B) The wild type and a mutated form (S232A) of StAR were used in the *in vitro* phosphorylation assay described in (A). The phosphorylated products were analyzed by SDS–PAGE and autoradiography. The results in (A) and (B) are representative of three independently performed experiments.**

StAR by PKA has no effect on ERK phosphorylation. The conditions in which the phosphorylation reaction takes place within the cells are critical. As for the case presented here, cholesterol is not only a component of the mitochondrial membrane where the reaction occurs, but it is also an endogenous ligand of StAR protein. Thus, our results indicate for the first time that StAR phosphorylation by a kinase different from PKA is modulated by the endogenous ligand of the protein.

3.4.5. Analysis of Ser232 as the phosphorylation site in phospho-StAR: Limited digestion by V8 endoprotease and separation of peptides in gel

An alternative approach to the use of proteins mutants is to analyze the phosphorylated peptides obtained by a limited protease digestion of a radioactively phosphorylated wild-type protein. The sequence of the protein has to be analyzed to choose a protease that renders putative phosphopeptides of different molecular sizes. This allows identification of the phosphorylated amino acid according to the molecular size of the radioactive peptide.

StAR includes within its sequence, cleavage sites for V8 endoprotease that are appropriate for this type of analysis. This protease hydrolyzes peptide bonds containing the α-carboxylic group of glutamic acid residues. StAR is an already known substrate of this protease. Sequence analysis of

StAR digestion using V8 endoprotease predicts the generation of several low molecular peptides with Ser232 as the only serine residue in an identifiable peptide of 6 kDa. Thus, we carried out an *in vitro* phosphorylation assay as described, followed by V8 proteolysis of phospho-StAR and analyzed the resulting peptides by electrophoresis and autoradiography. StAR (30 μg) phosphorylated in the presence of active His-tagged ERK1 and cholesterol was precipitated using chloroform:methanol. The resulting pellet was resuspended and proteolysis done in 100 m*M* phosphate buffer, pH 7.8 with a rate of enzyme:StAR of 1:30 at 37 °C overnight. The resulting peptides were resolved in polyacrylamide gels following the technique of Schagger and von Jagow (1987). This technique is suited for the identification of low molecular weight peptides and it uses 16.5% polyacrylamide mini gels with two different running buffers. The anode running buffer contains 200 m*M* Tris–HCl, pH 8.9 and the cathode running buffer contains 100 m*M* Tris–HCl; 100 m*M* Tricine, pH 8.25; 0.1% SDS. The electrophoresis was performed at room temperature for 1 h at 30 V in the concentration gel and for 4 h at 105 V in the resolution gel with the following low molecular weight markers: 1.06; 3.5; 6.5; 14.2; 17; 26.6 kDa (Sigma-Aldrich). Gels were then stained, dried, and exposed to autoradiography.

A unique radioactive band with a mass of 6 kDa, matching that of the expected fragment, was detected. This confirms that Ser232 is the target of ERK1/2 phosphorylation as anticipated by the *in vitro* phosphorylation assay already described (Fig. 10.4B).

To further test the identity of Ser232, 30 μg of phosphorylated and unphosphorylated StAR were subjected to SDS–PAGE. The gels were stained with Coomassie Brillant blue and the corresponding bands *were* excised from the gel for high pressure liquid chromatography (HPLC) analysis. Protein samples were partially digested with trypsin, and approximately 50 fractions were detected by peptide ionization. Due to technical issues and low ionization level in the peptides, the Ser232-containing peptide could not be detected in the samples. This situation is not unusual for several proteins of substantial molecular weight (the assay was performed in the Unity of Biochemistry and Analytic Proteomic, Institute Pasteur of Montevideo, Uruguay).

3.4.6. Effect of StAR phosphorylation by ERK1 on residue Ser232 on StAR cellular function

The physiological consequence of protein phosphorylation can be efficiently evaluated by transfection of wild type and different mutants of a given protein, in cells where it is possible to study a certain biological process.

In our case, we made use of two mutants of StAR protein, S232A and S232E, both generated by site directed mutagenesis of serine 232 to alanine

or glutamic acid, respectively. The structure and charges of glutamic acid resembles the phosphate residue present in the phosphorylated form of wild-type StAR.

Wild-type StAR and these two variants were transfected to MA-10 cells and steroid production evaluated. Steroid production enhanced by cAMP was inhibited in cells transfected with the non-phosphorylatable S232A mutant. The steroidogenic capacity was less affected by the replacement of Ser232 with Glu (S232E).

It follows from our results that a multi-kinase complex operates in the mitochondria for the regulation of steroid biosynthesis. The fine regulation of kinase activities results in the phosphorylation of a mitochondrial protein, affecting its function and finally, activating steroid biosynthesis.

4. Analysis of a Mitochondrial Acyl-CoA Thioesterase, Acot2 as a Phosphoprotein

Acot2, a mitochondrial protein, was identified in experiments aimed at identifying a protein involved in steroid synthesis through the release of AA. Those experiments led us to the isolation of a 43 kDa phosphoprotein (Dada *et al.*, 1991; Mele *et al.*, 1997; Neher *et al.*, 1982; Paz *et al.*, 1994). Further cloning and sequencing of the cDNA encoding the 43 kDa phosphoprotein revealed its primary structure (Finkielstein *et al.*, 1998) and the protein resulted 100% homologous to a mitochondrial-acyl-CoA thioesterase (Acot2) and 92.5% homologous to a cytosolic thioesterase (Acot1) (Lindquist *et al.*, 1998; Svensson *et al.*, 1998). Acot2 and Acot1 are members of a new acyl-CoA thioesterase family with very long chain and long chain acyl-CoA thioesterase activity (Lindquist *et al.*, 1998; Svensson *et al.*, 1998). The family includes four isoforms with different subcellular locations and a high degree of homology.

The sequence of Acot2 includes a mitochondrial leader peptide that targets this enzyme to the inner mitochondrial membrane (Finkielstein *et al.*, 1998; Stocco and Clark, 1996; Svensson *et al.*, 1998). In accordance with the postulated role of Acot2 in steroidogenesis, we detected the protein and its mRNA in all steroidogenic tissues including placenta and brain (Finkielstein *et al.*, 1998). Acot2 has an important role in the generation and export of arachidonic acid in the mitochondria of steroidogenic tissues (Castillo *et al.*, 2006). Interestingly, Acot2 takes part in a similar transduction pathway that regulates fatty acid export in heart mitochondria of diabetic rats (Gerber *et al.*, 2006).

Acyl-CoA thioesterases (EC 3.1.2.1. and EC 3.1.2.2.) are enzymes that catalyze the hydrolysis of CoA esters to free fatty acids and coenzyme A (CoA) (Hunt and Alexson, 2002; Yamada *et al.*, 2005). In the literature,

these enzymes have also been referred to as acyl-CoA hydrolases, acyl-CoA thioester hydrolases, and palmitoyl-CoA hydrolases. Although the roles of many acyl-CoA thioesterases in this protein family are not fully understood, they are considered to regulate intracellular levels of CoA esters, the corresponding free fatty acid and CoA and, consequently, cellular processes involving these compounds.

The Acot2 sequence contains a lipase serine motif as well as a Gly-Xaa-His motif close to the C-terminal region which has been shown to be required for the hydrolytic activity of the thioesterase. Antibodies raised against a synthetic peptide that includes the lipase serine motif blocked the activity of the enzyme (Finkielstein *et al.*, 1998).

The Acot2 sequence also contains consensus sites for different protein kinases such as PKA, PKC, CamK, and MAPKs. In agreement with this observation, Acot2 activity and its state of phosphorylation are dependent on hormone action. ACTH treatment of rat adrenal glands resulted in the appearance of multiple phosphorylated forms of the protein which were sensitive to acidic phosphatase treatment (Maloberti *et al.*, 2000). Acot2 activity is dependent on cAMP and PKA but its expression appears to be controlled by EGF (Castilla *et al.*, 2008) and not by cAMP (Cornejo Maciel *et al.*, 2005).

4.1. Purification of Acot2 to homogeneity preserving its biological activity

Acot2 was purified following standard procedures of ion exchange and gel filtration chromatography using homogenates of rat adrenal glands as starting material. Although the result was a 300-fold purification after several chromatography steps, SDS–PAGE analysis of the eluates still revealed the presence of several protein bands. Thus, purification to homogeneity of the protein was achieved by elution of Acot2 from an SDS–PAGE, a procedure that increased the purification to 13,000-fold. In general, protein elution from polyacrylamide gels is recommended in case other purification methods do not achieve further purification of the protein of interest.

The starting material in our protocol was a bioactive pool collected from a Superose-HPLC, but as a general rule, the samples should come from other purification steps and show as high purity as possible by conventional methods. The sample is first subjected to preparative electrophoresis, loading several lanes of an SDS–PAGE gel and running the electrophoresis under ordinary conditions. The lanes are cut into 2–4 mm slices and the slices extracted with 0.4 ml of 0.5% SDS at 4 °C for 16 h. The extracts are then saturated with urea in a final volume of 0.6 ml with the further addition of 40 μg of BSA. Sephadex G-10 equilibrated with 100 m*M* Tris–HCl, pH 8, was used as gel filtration resin to remove SDS. Aqueous urea (8 *M*, 0.1 ml) is applied to the column before sample loading. The protein

fractions from each column are collected and the content of the protein of interest evaluated in each fraction. The evaluation method is selected according with the known properties of the protein, that is, determination of biological or enzymatic activity or antibody recognition using dot blot or SDS–PAGE and western blot analysis. Regardless of the selected methodology, the eluates with higher content of the protein of interest are pooled and used for any further test, analysis or characterization. In our case, we made use of the known protein biological activity, that is, progesterone production in a cell-free assay, and of peptide mapping. Determination of the enzymatic activity is the most usual approach in case the function of protein of interest is known. After cloning, it became apparent that our protein was a thioesterase, therefore, we measured this activity and how it was regulated by hormones in our experimental model. Peptide mapping resulted also very convenient as it served for the generation of anti-peptide antibodies useful during cloning and identification of the protein by immunoblot or immunoprecipitation throughout the purification process (Paz *et al.*, 1994).

4.2. Determination of a thioesterase activity

In our case, analysis of enzymatic activity implies the determination of hydrolysis of the thioesther bond present in arachidonoyl-CoA. Acyl-CoA thioesterase activity is measured using 1-^{14}C-arachidonoyl-CoA (specific activity 51.6 mCi/mmol, concentration 0.02 mCi/ml) as substrate. The reaction was carried out using 0.1 μg of protein in 10 m*M* Hepes, 50 m*M* KCl, pH 7.4, and 15 μM of the substrate at 37 °C under vigorous shaking. Arachidonic acid released during the reaction was extracted from the aqueous phase with *n*-hexane and quantified by scintillation counting.

At this point, several facts pointed to the phosphoprotein nature of Acot2 (Fig. 10.5): (i) the purified protein evidenced as a double band after SDS–PAGE and silver staining; (ii) immunoblot analysis using an antibody raised against the N-terminal region of the thioesterase detected also a double band, indicating immunoreactivity of both components of the duplet; (iii) 2D electrophoresis resolved the protein in several spots. Taking together, these findings suggest that Acot2 is, in fact, a phosphoprotein.

4.3. Identification of Acot2 as a phosphoprotein

Metabolic labeling of cells with ^{32}P is the standard method for monitoring the level of protein phosphorylation. The combination of metabolic labeling with specific immunoprecipitation and one- or two-dimensional gel electrophoresis is a powerful tool for the identification of phosphoproteins. Other methods useful for the investigation of phosphoproteins include

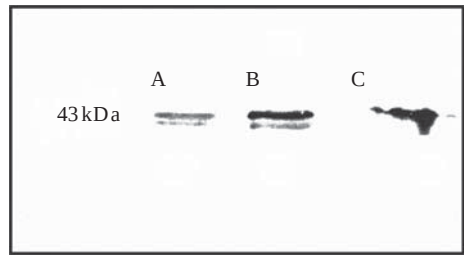

Figure 10.5 Analysis of Acot2. (A) SDS–PAGE and silver staining of elutes of Acot2 from SDS–polyacrilamide gels. (B) Western blot analysis using anti-peptide antibodies. The double band suggests that there is only one protein in different phosphorylation states. (C) 2D-electrophoresis and western blot. The development of several spots confirms the multiple phosphorylation of the protein.

in vitro phosphorylation and dephosphorylation of purified proteins or from recombinant proteins expressed in bacteria.

In vitro phosphorylation of a given protein is useful for determining its behavior as substrate for different kinases. This can be done by using a particular kinase, the purified protein and [γ-^{32}P]-ATP as donor. Since the protein has to be in its dephosphorylated form, purification from the tissue or cells under basal (non-activated) conditions or production in bacteria by recombinant cDNA procedures is a prerequisite. The incorporation of the radioactive phosphate into the protein is evaluated in an aliquot of the reaction mixture by scintillation counting of TCA or acetone-precipitated proteins, autoradiography of SDS–polyacrilamide gels or after trapping the sample in phosphocellulose paper and thorough washing before counting. In the case of Acot2 phosphorylation by PKA, the reaction is performed in a total volume of 40 μl of a reaction buffer that contains 50 m*M* phosphate buffer, pH 6.5; 20 m*M* MgCl; 5 μg of Acot2 and 2 units of the catalytic subunit of PKA during 10 min at 30 °C. Once the reaction is completed, the mixture is subjected to SDS–PAGE, and the resulting gel stained with Coomassie Brilliant Blue, dried and exposed to autoradiography films or to phosphorimager screens.

The dephosphorylation assay relies on phosphatases that act on the phosphorylated form of the protein. This assay is usually performed in combination with 2D-electrophoresis. In steroidogenic cells, the phosphorylation of Acot2 is under the control of hormone action. Thus, confirmation of the existence of different phoshorylation states of the thioesterase was performed using Acot2 partially purified from adrenal glands of ACTH-treated animals as substrate and potato acid phosphatase as enzyme. The reaction mixture contains 120 mIU of the enzyme and 5–20 μg protein in 10 m*M* Tris–HCl pH 6.5 buffer (total volume 9 μl) and the reaction proceeds during 30 min at 37 °C. The sample is then subjected to

two-dimensional gel electrophoresis and immunoblot analysis. Potato acid phosphatase treatment of Acot2 was effective in reducing the number of spots detected after 2D electrophoresis. While the starting material (the phosphorylated form of the enzyme) shows about seven different spots, phosphatase treatment renders the thioesterase as a double spotted protein, thus confirming protein phosphorylation as an important posttranslational modification of Acot2.

Hormone action on steroidogenic tissues does not affect the expression of Acot2. Therefore, Acot2 constitutes a good example of the importance of posttranslational modifications by protein phosphorylation in mitochondria that trigger the protein's full activity. Finally, Acot2 activation depends on substrate availability and its covalent modification.

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