Protein Modifications Regulate the Role of 14-3-3-γ Adaptor Protein in cAMP-induced Steroidogenesis in MA-10 Leydig Cells*

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Background: The interaction between 14-3-3-γ and steroidogenic acute regulatory protein (STAR) delays maximal steroidogenesis in a pattern opposite to 14-3-3-γ homodimerization.

Results: The phosphorylation and acetylation of 14-3-3-γ on residues Ser58 and Lys49 increase STAR binding.

Conclusion: The phosphorylation and acetylation of 14-3-3-γ regulate dimerization, STAR binding, and steroidogenesis.

Significance: Blocking amino acid modifications on 14-3-3-γ increases steroid production.

The 14-3-3 protein family comprises adaptors and scaffolds that regulate intracellular signaling pathways. The 14-3-3-γ isoform is a negative regulator of steroidogenesis that is hormonally induced and transiently functions at the initiation of steroidogenesis by delaying maximal steroidogenesis in MA-10 mouse tumor Leydig cells. Treatment of MA-10 cells with the cAMP analog 8-bromo-cAMP (8-Br-cAMP), which stimulates steroidogenesis, triggers the interaction of 14-3-3-γ with the steroidogenic acute regulatory protein (STAR) in the cytosol, limiting STAR activity to basal levels. Over time, this interaction ceases, allowing for a 2-fold induction in STAR activity and maximal increase in the rate of steroid formation. The 14-3-3-γ/STAR pattern of interaction was found to be opposite that of the 14-3-3-γ homodimerization pattern. Phosphorylation and acetylation of 14-3-3-γ showed similar patterns to homodimerization and STAR binding, respectively. 14-3-3-γ Ser58 phosphorylation and 14-3-3-γ Lys49 acetylation were blocked using trans-activator of transcription protein.

The 14-3-3 protein family consists of adaptors and scaffolds that regulate cellular pathways by affecting signal transduction and protein localization and by altering protein modifications. Seven isoforms of the 14-3-3 family are expressed in mammary gland. All of the isoforms are highly helical because the 14-3-3 monomers contain nine α-helices. The N termini of 14-3-3 proteins contain the dimerization domain where salt bridges form between two monomers to form a dimer. The affinity of 14-3-3 isoforms for homo/heterodimerization varies, but each 14-3-3 isoform exhibits strong preference for dimer formation (1–3). The C termini of these proteins contain target binding sites (4). Because of high homology among 14-3-3 C termini, some groups have posited that 14-3-3 functions are redundant and that one can replace another (5–8).

Recent studies have shed light on the isoform specificity of 14-3-3 family members for certain targets in different tissues (9). Computational analysis of the networks for each 14-3-3 isoform revealed that they are distinct, and multiple knockdown studies resulted in severe alterations in cell metabolism, proliferation, protein trafficking, cell division, etc., providing further evidence of 14-3-3 isoform and tissue specificity (4, 10–12). Moreover, recent studies have indicated that 14-3-3 isoforms are highly variable, and post-translational modifications (PTMs) influence target/isoform specificity (for reviews, see Refs. 13–19). Moreover, recent studies have indicated that 14-3-3 monomers are also functional (20); 14-3-3-e-sv is a monomeric form of 14-3-3-e that lacks the exon encoding the N-terminal dimerization domain but remains functional and promotes cell survival similar to intact 14-3-3-e (21), and 14-3-3-γ S58N monomers interact with the scaffold protein kinase suppressor of Ras in the same manner as 14-3-3-γ dimers (22). The majority, but not all, of 14-3-3 targets contain the 14-3-3 binding motifs RXSXP (mode I) and RX(Y/F)XpSXP (mode II) (11, 21) where pS

The abbreviations used are: PTM, post-translational modification; 8-Br-cAMP, 8-bromo-cAMP; IP, immunoprecipitation; phos-tag, phosphate-binding tag; STAR, steroidogenic acute regulatory protein; TAT, trans-activator of transcription protein.

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is phosphoserine as well as a recently discovered consensus in the C-terminal domain of targets, -(pS/pT)X₁₋₂-CO₂H (mode III) where pT is phosphothreonine and X is not Pro (23, 24). Mutational analysis was performed to identify critical residues for protein dimerization and target binding within the 14-3-3 structure, and the results suggested that if the target protein contains more than one suboptimal 14-3-3 motif the affinity of 14-3-3 for the target is increased by 30-fold, which can permit interactions of the monomeric version with its targets (23–25).

We recently demonstrated that 14-3-3γ is part of the transduceosome (23), a protein complex that mediates the import of cholesterol from cytosolic sources into mitochondria, which is the rate-limiting step in steroidogenesis. Cholesterol is metabolized in the inner mitochondrial membrane by CYP11A1 to pregnenolone, the precursor of all steroids (26). It is at the transduceosome where the hormone-induced steroidogenic acute regulatory protein (STAR) accelerates cholesterol import into mitochondria (27). We recently reported that STAR is a target of the 14-3-3γ isoform (26). STAR contains three suboptimal 14-3-3 motifs on critical structure sites and therefore binds 14-3-3γ monomers with high affinity. 14-3-3γ and STAR have similar transcription and translation profiles in MA-10 mouse Leydig tumor cells (26), and pre-existing mRNA encoding both proteins is translated upon treatment with the membrane-permeable cAMP analog 8-Br-cAMP. This compound is used to mimic endogenous cAMP, the secondary messenger of gonadotropin luteinizing hormone and the main activator of Leydig cell steroidogenesis. cAMP treatment also disrupted 14-3-3γ dimers, and the monomeric form went on to interact with a serine residue (Ser194) on STAR (28). This residue is a phosphorylation site for PKA. STAR phosphorylation at Ser194 induces STAR activity by 2-fold, which is required for maximal steroidogenesis. Ser194 mutations are one of the causes of congenital adrenal lipid hyperplasia, a condition in which a lack of sufficient steroidogenesis causes cholesterol accumulation and adrenal cortex cells undergo hyperplasia and apoptosis (26). By locking phosphorylation of this site, 14-3-3γ is able to retain STAR in the cytosol under basal activity, thus delaying its effect on mitochondrial steroidogenesis. After 2 h, 14-3-3γ dissociates from STAR and homodimerizes, allowing STAR to induce steroidogenesis at a maximal rate (29, 30). As STAR binding and 14-3-3γ homodimerization have opposite patterns, we sought to identify potential regulatory mechanisms for the time-sensitive function of 14-3-3γ in steroidogenesis.

PTMs of 14-3-3 proteins are important regulators of their functions. Phosphorylation can lead to self-dimerization (26) or interactions with specific targets (for a review, see Ref. 20). For instance, Ser266 phosphorylation of 14-3-3ζ, -β, and -γ by PKA leads to interactions with sphingosine (31) and regulates 14-3-3 oligomerization (32). Ser185 phosphorylation of 14-3-3β, -ζ, and -α (22) as well as Thr233 phosphorylation of 14-3-3ζ and -τ facilitates interactions with JNK (33). In addition, Choudhary et al. (34) reported that the “lysinic acetylome” could acetylate all 14-3-3 isoforms on Lys49. Such a PTM was not identified in 14-3-3γ or -β. Indeed, acetylation of key lysine residues in vivo indicates a novel regulatory mechanism of 14-3-3 function as this residue is located in the 14-3-3 monomer amphipathic groove through which they bind to their targets. Furthermore, Uhart and Bustos (13) reported cross-talk between 14-3-3 protein acetylation and phosphorylation in which acetylation of 14-3-3ζ on Lys49 inhibits the phosphorylation-dependent interactions of this protein with specific targets (35). In the present study, we investigated the effect of 14-3-3γ acetylation and/or phosphorylation on STAR binding and 14-3-3γ homodimerization during steroidogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatments, and Steroid Measurement—MA-10 mouse Leydig tumor cells (kindly provided by Dr. M. Ascoli, University of Iowa) were maintained in DMEM/Ham’s nutrient mixture F-12 supplemented with 5% FBS, 2.5% horse serum, and 1% penicillin and streptomycin at 37°C and 3.7% CO₂. For the time course experiments, cells were incubated in serum-free medium and supplemented with 1 μM 8-Br-cAMP (Enzo Life Sciences). The time course treatment was carried out for 30, 60, or 120 min as indicated in the figures. When the trans-activator of transcription protein (TAT) peptide was used, 1 × 10⁵ MA-10 cells were cultured, and after 24 h, the medium was replaced by DMEM/nutrient mixture F-12 without serum supplemented with 250 nM TAT peptide for 90 min. Progesterone or testosterone levels were measured by RIA in triplicate using specific antibodies against rabbit and sheep, respectively (MP Biomedicals), and a β counter (LS5801, Beckman Coulter). Protein levels in each well were measured with 1 N NaOH extraction buffer and the Bradford dye assay (Bio-Rad) using a Beckman Coulter spectrophotometer.

Protein Extraction—MA-10 cells (6 × 10⁵/well) were cultured in 6-well gelatin-coated plates in triplicate for 24 h. A cAMP time course treatment was carried out. Cells were washed twice in 4 ml of 1× PBS and harvested. Proteins were extracted using radioimmune precipitation assay lysis buffer (Cell Signaling Technology), and concentrations were measured using the Bradford dye assay (Bio-Rad) and a Beckman Coulter spectrophotometer.

Dephosphorylation Assay—MA-10 cell lysate was extracted, and 15 μg of lysate was incubated at 37°C for 1 h in 50 mM Tris-HCl buffer (pH 9.0; 0.20 ml) containing 1.0 mM MgCl₂, 50 μg of protein, and 3.3 units of calf alkaline phosphatase (New England Biolabs). The reaction was stopped by the addition of Laemmli loading buffer at room temperature. Samples were store at −20°C until further use.

Phosphate-binding Tag Acrylamide Electrophoresis and Immunoblot Analysis—SDS-polyacrylamide gels were prepared using 8.2% acrylamide and 25 nM phosphate-binding tag (phos-tag; Wako Pure Chemical Industries, Ltd.) after optimization. Samples were solubilized in Laemmli buffer, heated at 65°C for 5–10 min, and loaded directly onto phos-tag acrylamide gels. Proteins were separated at 90 V for 3.5 h. The gels were then washed in wash buffer containing 10 mM EDTA to remove the phos-tags for 20 min and then with wash buffer without EDTA for 10 min and electrotransferred onto PVDF membranes. Membranes were blocked using 10% milk and blotted with a 1:1000 dilution of 14-3-3-γ-specific antibody (Santa Cruz Biotechnology) at 4°C overnight. The following day, the membranes were washed with 1× Tween 20-TBS twice at room temperature and incubated with secondary anti-rabbit IgG
HRP-linked antibody (Cell Signaling Technology) for 1 h with shaking at room temperature to detect 14-3-3γ. Membranes were developed using ECL streptavidin-horseradish peroxidase conjugate (GE Healthcare) and a luminescent image analyzer (LAS-4000, GE Healthcare).

Cross-linking—MA-10 cells (1 × 10⁶/Petri dish) were plated in gelatin-coated 100-mm Petri cell culture dishes overnight to reach 90% confluence. Medium was replaced with Dulbecco’s modified Eagle’s limiting medium supplemented with 10% FBS and 105 mg/liter photo-leucine and 30 mg/liter photo-methionine (Thermo Scientific). Cells were incubated for 22 h followed by cAMP time course treatment. Medium was decanted and replaced with 4 ml of 1× PBS. Cross-linking was performed immediately using a 3UV lamp (UVP, LLC) for 16 min at 365 nm at a distance of 1 cm from the Petri dish surfaces.

Co-immunoprecipitation—Co-immunoprecipitation was performed using a Dynabeads Co-Immunoprecipitation kit (Invitrogen). The antibody coupling process was performed using anti-acetyl-lysine or anti-phosphoserine (both from Abcam) antibodies following the manufacturer’s recommendations, which yielded 10 mg/ml antibody-coupled beads. Cross-linked protein lysates of MA-10 cells were harvested according to the manufacturer’s recommendations. Proteins were collected in extraction buffer A (containing 1× immunoprecipitation buffer, 1 mM NaCl, and protease inhibitor tablets without EDTA), and 0.2 mg of protein was precipitated with 150 μl of 10 mg/ml antibody-coupled beads rotating at 4 °C for 1 h.

Mass Spectrometry Analysis—MA-10 cells were subjected to 8-Br-cAMP treatment for different time points as indicated in the figures. Cells lysates were pulled down during co-immunoprecipitation using 14-3-3γ antibody. Samples were subjected to 65 °C heat for 10 min to dissociate the protein complexes and then separated on phos-tag gels, which were processed with silver staining according to the manufacturer’s manual (Invitrogen). Lanes/bands corresponding to different time points of cAMP treatment were cut out, subjected to in-gel digestion, and analyzed via mass spectrometry. Briefly, nanoflow reversed-phase liquid chromatography was performed with an Agilent 1100 LC system (Agilent Technologies) coupled online to a linear ion trap mass spectrometer (LTQ, Thermo Scientific). Reversed-phase chromatography was performed with an Agilent 1100 LC system over 20 min with 98% solvent A (0.1% (v/v) formic acid in water) and 2% solvent B for 110 min at a constant flow rate of 250 nl/min. Each full MS scan was followed by seven MS/MS scans, and the most abundant peptide molecular ions were dynamically selected for collision-induced dissociation using a normalized collision energy of 36%. The collision-induced dissociation spectra were searched against a non-redundant human protein database using SEQUEST (Thermo Scientific).

Immunoblot Analysis—MA-10 cell lysates were cross-linked and harvested. A sample of the lysate was kept as a control, whereas the rest was subjected to precipitation with 14-3-3γ antibody. The samples were then separated by 8.25% phos-tag acrylamide electrophoresis as described above. The corresponding bands of each sample at different molecular weights were cut and silver-stained (Invitrogen) according to the manufacturer’s manual and analyzed via mass spectrometry as described previously (13), or after blue native PAGE, proteins were further transferred to a PVDF membrane for detection of 14-3-3γ and -ε in native mitochondrial complexes using 1:1000 dilutions of specific antibodies (Santa Cruz Biotechnology). The membranes were stripped using 8 ml of Restore Plus Western blot Stripping Buffer (Thermo Scientific) and reblotted using a 1:1000 dilution of cytochrome c oxidase IV antibody (Abcam).

In-cell IP and Confocal Microscopy—MA-10 cells (1 × 10³/well) were cultured in 96-well glass bottom dishes (Fluorodish, World Precision Instruments) in triplicate and incubated overnight. Cells were treated in a time course manner with cAMP as described above. Cells were fixed using 3.7% formaldehyde for 15 min in 37 °C, washed with 1× PBS twice at room temperature, and permeabilized with 10% Triton X-100 for 1 min at room temperature. In-cell IP was performed using the Duolink II Red Starter kit (Olink Bioscience) according to the manufacturer’s recommendations. Primary antibodies were used in combinations of 14-3-3γ (anti-mouse and anti-rabbit, 1:150 dilutions; Santa Cruz Biotechnology), and 14-3-3γ (anti-mouse, 1:150 dilution), and STAR (anti-rabbit, 1:150 dilution; kindly provided by Dr. B. Hales, University of Southern Illinois (36)); all incubations were carried out overnight at 4 °C. Mitochondria and nuclei were stained using Mito-ID and Hoechst (both at 1:250; Enzo Life Sciences), respectively, for 30 min at 37 °C. Cells were washed and maintained in ultrapure water. An Olympus Fluoview FV1000 laser confocal microscope equipped with a 100× lens was used to detect 14-3-3γ homodimers or interaction with STAR. Z stacks were captured from the bottom to the top of the cell nucleus for a minimum of 40 cells. The sum of signals in all Z stacks was measured by Olink software and normalized to the total number of cells to obtain a signal/cell ratio.

Sequence Alignment—ClustalW software was used to align the sequences of 14-3-3γ, -ε, and TAT fusion peptides.

Peptide Design—Peptides were designed to contain an 11-mer of the HIV-1 TAT followed by a glycine residue conjugated to different regions of 14-3-3γ as follows: amino acids 1–10, the N-terminal dimerization domain (TAT-14-3-3γ 1–10; YGRKKRRQRRRGMVDREQLVQKARLA) or amino acids 45–60 (TAT-14-3-3γ 45–60; YGRKKRRQRRRSVAYKNVVGARRSW); TAT-14-3-3γ 45–60 containing the Lys⁴⁹ acetylation site but with Ser⁵⁸ mutated to a glycine residue (TAT-14-3-3γ Lys⁴⁹; YGRKKRRQRRRSVAYKNVVGARRSW); TAT-14-3-3γ 45–60 containing the Lys⁴⁹ acetylation site but with Ser⁵⁸ mutated to a glycine residue (TAT-14-3-3γ Lys⁴⁹; YGRKKRRQRRRSVAYKNVVGARRSW); TAT-14-3-3γ 45–60 containing the Lys⁴⁹ acetylation site but with Ser⁵⁸ mutated to a glycine residue (TAT-14-3-3γ Lys⁴⁹; YGRKKRRQRRRSVAYKNVVGARRSW); TAT-14-3-3γ 45–60 containing the Lys⁴⁹ acetylation site but with Ser⁵⁸ mutated to a glycine residue (TAT-14-3-3γ Lys⁴⁹; YGRKKRRQRRRSVAYKNVVGARRSW); TAT-14-3-3γ 45–60 containing the Lys⁴⁹ acetylation site but with Ser⁵⁸ mutated to a glycine residue (TAT-14-3-3γ Lys⁴⁹; YGRKKRRQRRRSVAYKNVVGARRSW); TAT-14-3-3γ 45–60 containing the Lys⁴⁹ acetylation site but with Ser⁵⁸ mutated to a glycine residue (TAT-14-3-3γ Lys⁴⁹; YGRKKRRQRRRSVAYKNVVGARRSW).
nal Greece. MA-10 cells were treated with these peptides at 250 nM after optimization for 90 min.

Oil Red O Staining and Microscopy—MA-10 cells (1 × 10^5) were plated on 6-well gelatin-coated culture dishes and kept on growth medium overnight. The next day, the medium was replaced with medium without serum and with or without 250 nM TAT-14-3-3 Lys^69 or TAT-14-3-3 Ser^58 for 90 min followed by 1 mM 8-Br-cAMP (Enzo Life Sciences) treatment for 120 min. Cells were washed in 1× PBS and fixed using 3.7% formaldehyde solution for 20 min in 37 °C. Filtered oil red O solution (Sigma-Aldrich) was incubated on the cells for 5 min at room temperature. Cells were further washed with 60% isopropanol alcohol for 5 min, dried completely, and maintained in ultrapure water. Images were captured using an Olympus inverted microscope with 40× lens. Then oil red O staining was removed by incubating cells with 100% isopropanol alcohol solution for 10 min at room temperature while shaking. UV light at 520 nm was used to measure lipid levels in each cell.

Cholesterol Detection Assay—MA-10 cells (1 × 10^5) were plated on 6-well glass bottom dishes (Fluorodish) and kept on growth medium overnight. The next day, the medium was replaced with medium without serum and with or without 250 nM TAT-14-3-3 Lys^69 or TAT-14-3-3 Ser^58 for 90 min followed by 1 mM 8-Br-cAMP treatment for 120 min. After the treatment period, medium was removed, cells were washed with 1× PBS, and cholesterol staining was performed using the Cholesterol Cell-Based Detection Assay kit, which applies filipin staining (Cayman Chemicals).

Statistical Analysis—All experiments were performed in triplicate in three separate cell passages. All data shown are means ± S.E., or data are representative from three independent experiments as indicated in the figure legends. RIA was performed in triplicate for each passage. Statistical analysis was performed by two-tailed unpaired t tests using Prism v5.0 (GraphPad Software). In-cell IP was performed on a minimum of 50 cells per passage. Statistical analysis was performed by two-tailed unpaired t tests to compare the protein interactions at each time point between control cells and cells treated with control peptide, whereas one-way analysis of variance test was performed to compare protein interactions at each time point between the control group (control cells and cells treated with control peptide) versus the cells treated with TAT-14-3-3 Ser^58 or TAT-14-3-3 Lys^69 using Prism v5.0 (GraphPad Software). * represents p ≤ 0.05, ** represents p ≤ 0.01, and *** represents p ≤ 0.001.

RESULTS
Dimerization Versus Target Binding State of 14-3-3γ Regulates Steroidogenesis—To confirm the previous findings indicating that 14-3-3γ homodimerization and interactions with STAR regulate steroidogenesis levels at 0 and 120 min or 15–60 min post-cAMP treatment, respectively (37), dimerization or target binding sites of 14-3-3γ were competed out using TAT-14-3-3γ 1–10 or TAT-14-3-3γ 219–236, respectively (Fig. 1A). These peptides were incubated with MA-10 cells for 90 min during which time they penetrate through cell membranes (26) and compete against dimerization of 14-3-3γ through its N-terminal amino acids 1–10 or against the 14-3-3γ target binding site through amino acids 219–236 at its C-terminal domain. MA-10 cells were subsequently treated for the indicated time points with 8-Br-cAMP (1 mM) to induce steroidogenesis. Progesterone levels in the media were measured by RIA. The results obtained confirmed our previous data showing that if the 14-3-3γ dimerization site is blocked then maximally stimulated cAMP-induced steroid formation is further increased at 120 min (Fig. 1B). However, when STAR binding was blocked, steroid levels were only increased at 30–60 min post-cAMP treatment (Fig. 1C), suggesting that 14-3-3γ dimerization and STAR binding are tightly regulated and function consecutively to regulate steroidogenesis. As 14-3-3 proteins have a high degree of homology, these peptides were aligned with sequences of the 14-3-3γ and −ε (Fig. 1D), the two isoforms identified to play a role in steroidogenesis. The sequence alignment indicates that amino acids 1–10 of 14-3-3γ and −ε are highly variable; therefore, the TAT-14-3-3γ 1–10 fusion peptide specifically blocks 14-3-3γ homodimerization. Conversely, amino acids 219–236 of both proteins are identical so the TAT-14-3-3γ 219–236 fusion peptide can also block the interactions of 14-3-3ε with its targets. However, because 14-3-3ε only regulates steroidogenesis after 120 min of treatment with 8-Br-cAMP, the increase in steroid production at earlier time points is specific to 14-3-3γ protein (26).

14-3-3γ Phosphorylation and Acetylation Have Opposite Patterns—Phosphorylation and acetylation of 14-3-3γ protein in MA-10 cells were measured. Photoactivatable amino acids were used for cross-linking to strengthen the interactions of protein kinases or acetylases with the protein after each cAMP stimulation (27). This made any modifications irreversible throughout the experiment. The MA-10 cell lysates were then precipitated with either phosphoserine or acetyl-lysine antibodies coupled to Dynabeads to isolate the pools of phosphorylated or acetylated proteins, respectively. Immunoblot analysis using a 14-3-3γ-specific antibody was performed to determine the levels of phosphorylated (Fig. 2A) or acetylated 14-3-3γ protein (Fig. 2B). Because 14-3-3γ expression is induced by hormones and cAMP (26, 38), the total levels of 14-3-3γ were also measured at the same time points (Fig. 2C), and levels of phospho-14-3-3γ or acetyl-14-3-3γ were normalized to the total protein levels at the corresponding time points (Fig. 2, D and E). The results indicate that phosphorylation and acetylation of 14-3-3γ have opposite patterns. At 0 and 120 min, 14-3-3γ phosphorylation was at its highest, whereas acetylation peaked at 60 min. Moreover, the phosphorylation pattern of 14-3-3γ resembles the dimerization pattern of this protein (26), and the acetylation pattern of 14-3-3γ has similarities with the 14-3-3γ/STAR interaction pattern (26). To further confirm these data, MA-10 cells were treated with 8-Br-cAMP for different times, and cell lysates were harvested at each time point and divided into two samples. One sample was kept in its native state, and the other sample was subjected to a dephosphorylation assay using alkaline phosphatase. Phos-tag acrylamide electrophoresis was used to separate the phosphoproteins. The phos-tag molecule is an alkoxide-bridged dinuclear Mn^{2+} that...
14-3-3 Y Integrates into the Acrylamide Gels During Preparation. Because of the vacancies on Mn²⁺ metal ions, this molecule captures the phosphomonoester dianions bound to proteins loaded on the gel as a bridging ligand and leads to a mobility shift of phosphorylated proteins in SDS-PAGE (39). Using anti-14-3-3 antibody, the level of phosphorylated 14-3-3 at each time point was identified as separate bands on the gel. The levels of 14-3-3 phosphorylation at each time point were determined by comparing the number of bands recognized by the 14-3-3 antibody in the native state lysate (Fig. 2F, lanes a) or the dephosphorylated lysate (Fig. 2F, lanes b). The thick band observed in lane b is the alkaline phosphatase used for dephosphorylation, the concentration of which is so high that it can be detected without any antibody. These results indicate a lower number of bands corresponding to phospho-14-3-3 at 15, 30, and 60 min of 8-Br-cAMP treatment (Fig. 2G).

14-3-3 Y Homodimers Are Phosphorylated, and Monomers Are Acetylated—Phosphorylation of Ser 58 on 14-3-3 has been shown to have a role in its self-dimerization (31), and acetylation of 14-3-3 on Lys49 inhibited the phosphorylation-dependent interactions of this protein with its targets (35). In this study, we developed TAT peptides to compete with Ser58 phosphorylation (TAT-14-3-3 Ser58) and Lys49 acetylation (TAT-14-3-3 Lys49) sites to further assess their function in steroidogenesis (Fig. 3A). As Lys49 and Ser58 are located near each other, developing TAT peptides containing either Ser58 or Lys49 was not feasible. Therefore, both TAT peptides cover amino acids 45–60, but TAT-14-3-3 Lys49 contains a Gly residue instead of Ser58 and thus cannot efficiently compete with the Ser58 phosphorylation site, whereas TAT-14-3-3 Ser58 contains a Gly instead of Lys49 so that it can compete only with Ser58 phosphorylation (Fig. 3A). Moreover, a control peptide with a Gly residue instead of both Lys49 and Ser58 was developed to serve as a control.

MA-10 cells were incubated with TAT-14-3-3 Gly49,Gly58 (control), TAT-14-3-3 Ser58, or TAT-14-3-3 Lys49 peptide for 90 min followed by treatment with 1 mM 8-Br-cAMP treatment as indicated. 14-3-3 homodimerization was measured at each time point by in-cell IP using the Duolink system and compared to the corresponding levels in control cells (Fig. 3, A)

Figure 1. 14-3-3 Y Dimerization and STAR Binding Regulate Steroidogenesis in a Time-Dependent Manner. A, TAT peptides were conjugated to the N-terminal dimerization or C-terminal target-binding domain of 14-3-3, and the sequences are shown. MA-10 cells were treated with TAT-14-3-3 1–10 to block 14-3-3 Y dimerization, including high affinity homodimerization (B), or TAT-14-3-3 219–236 to block 14-3-3 target binding, including STAR binding (C). Progesterone levels were measured by RIA at each time point after cAMP treatment as indicated. Results shown are means ± S.E. (error bars) from three independent experiments performed in triplicate. D, amino acid sequence homologies of the γ and ε 14-3-3 isoforms, where asterisks indicate amino acids conserved in all three sequences and periods indicate conserved amino acids in two out of the three sequences shown. * p ≤ 0.05.
The results obtained indicated that if the Ser58 phosphorylation of 14-3-3/H9253 is competed out by the TAT-14-3-3/H9253 Ser58 peptide then 14-3-3/H9253 dimerization is induced with a more pronounced effect at 120 min (Fig. 3, A–C and E), the time point at which there is high phosphorylation (Fig. 2, A, C, G, and F). Under these conditions the control fusion peptide did not alter the endogenous 14-3-3γ homodimerization.

Blocking acetylation of 14-3-3γ on Lys49 reversed the 14-3-3γ homodimerization pattern (Fig. 3, A, B, D, and E) when acetylation was shown to be highest (Fig. 3, D and E). Therefore, Ser58 phosphorylation and Lys49 acetylation are required for 14-3-3γ dimerization, and the levels of these modifications balance the homodimer and monomer states of 14-3-3γ in a time-dependent manner.

**Ser58 Phosphorylation Is Required for 14-3-3γ/STAR Interactions, but Lys49 Acetylation Induces Such Interactions**—Control TAT-14-3-3γ Gly49, Gly58, TAT-14-3-3γ Ser58, or TAT-14-3-3γ Lys49 were used to study the 14-3-3γ/STAR interaction in MA-10 cells by in-cell IP. The results indicated that whereas the control peptide does not alter STAR-14-3-3γ interactions (Fig. 4A), the TAT-14-3-3γ Ser58 peptide induced such interactions (Fig. 4B).

**Acetylation**—Acetylation was shown to be required for 14-3-3γ/STAR interactions (Fig. 4C). Blocking acetylation of 14-3-3γ on Lys49 reversed the 14-3-3γ homodimerization pattern (Fig. 3, A, B, D, and E) when acetylation was shown to be highest (Fig. 3, D and E). Therefore, Ser58 phosphorylation and Lys49 acetylation are required for 14-3-3γ dimerization, and the levels of these modifications balance the homodimer and monomer states of 14-3-3γ in a time-dependent manner.

**Ser58 Phosphorylation Is Required for 14-3-3γ/STAR Interactions, but Lys49 Acetylation Induces Such Interactions**—Control TAT-14-3-3γ Gly49, Gly58, TAT-14-3-3γ Ser58, or TAT-14-3-3γ Lys49 were used to study the 14-3-3γ/STAR interaction in MA-10 cells by in-cell IP. The results indicated that whereas the control peptide does not alter STAR-14-3-3γ interactions (Fig.

**FIGURE 2. 14-3-3γ phosphorylation and acetylation pattern during steroidogenesis.** MA-10 cells were treated with 1 mM 8-Br-cAMP for the indicated times, cross-linked using photo-Leu/Met and UV light exposure, and precipitated using phospho-Ser (A) and acetyl-Lys (B) antibodies. A 14-3-3γ antibody was used to measure 14-3-3γ levels in the pool of phosphorylated or acetylated MA-10 cell lysate in three distinct cell passages (n = 3). Immunoblot (IB) signals from three independent experiments were quantified and normalized to total levels of 14-3-3γ at each time point, compared with levels of the loading control GAPDH (C), and graphed (D and E). F, MA-10 cells were treated with 1 mM 8-Br-cAMP for the indicated times. Similar protein concentrations at each time point were submitted to acrylamide dephosphorylation assays, and equal levels were maintained in their native state. The proteins were electrophoresed on phos-tag acrylamide gels. Using a 14-3-3γ antibody, the differentially phosphorylated 14-3-3γ proteins at each time point in the native state were identified by comparison with the dephosphorylated bands of the corresponding time point. The results are representative of three independent experiments. G, mass spectrometry analysis of the 14-3-3γ-enriched protein pool separated based on phosphorylation levels on phos-tag gels. All data shown are means ± S.E. (error bars), and data are representative from three independent experiments. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001. A.U., arbitrary units.
The STAR binding pattern is reversed if Ser58 phosphorylation is blocked (Fig. 4, A and B), showing a complete dissociation between the two proteins at 60 min. However, if Lys49 acetylation was blocked, STAR interactions remained similar to basal levels, and the 8-Br-cAMP-dependent induction of these interactions at 30–60 min was no longer observed (Fig. 4, A, B, D, and E). Although these protein/protein interactions did not show a significant reduction, there was a trend of decrease, suggesting that 14-3-3γ/STAR interactions may be a consequence of dissociation of 14-3-3γ homodimers. Therefore, 14-3-3γ phosphorylation on Ser58 is required for basal interactions between this protein and STAR, whereas Lys49 acetylation induces such interactions upon cAMP treatment. The decrease in 14-3-3γ/STAR interactions at 120 min following 8-Br-cAMP treatment observed in control cells is not regulated by either modification; rather, it is likely to be a consequence of 14-3-3γ homodimerization at this time point.

Blocking 14-3-3γ Modifications Induces Steroidogenesis—To assess the physiological impact of blocking 14-3-3γ modifications on steroidogenesis, MA-10 cells were incubated with either TAT-14-3-3γ Ser58 or TAT-14-3-3γ Lys49, and the levels of steroids were measured following cAMP treatment for the indicated time points. The results indicated that blocking Ser58 phosphorylation of 14-3-3γ induced steroidogenesis with a more pronounced effect at 120 min when there is high phosphorylation in control cells (Fig. 5A). If Lys49 acetylation of 14-3-3γ was blocked, steroidogenesis was also induced, but this effect was most significant at 60 min when 14-3-3γ acetylation was highest in control cells (Fig. 5B).
These results confirm the role of 14-3-3γ modifications in cAMP-dependent steroidogenesis.

Blocking 14-3-3γ Modifies Reduces Cholesterol Accumulation—Increased steroid formation should lead to lower levels of free cholesterol available for steroidogenesis in MA-10 cells. Therefore, changes in lipid droplets (sites of storage of cholesterol and cholesteryl esters) and free cholesterol levels were evaluated in the presence of TAT-14-3-3γ fusion peptides using oil red O and filipin staining, respectively. Treatment of MA-10 cells with 1 mM 8-Br-cAMP resulted in a slight but significant reduction of lipid storage (Fig. 6, A and B) and a pronounced reduction in the levels of free cholesterol (Fig. 6C). However, upon addition of the TAT-14-3-3γ Lys49 or TAT-14-3-3γ Ser58, there was a significant reduction in MA-10 cell lipid droplet oil red O staining (Fig. 6, A and B) and free cholesterol levels in MA-10 cells (Fig. 6C), indicating that the peptides reduced 14-3-3γ/STAR interaction, and the free STAR could then deplete free cholesterol and therefore the lipid content in a manner parallel to increased steroid formation.

DISCUSSION

We recently identified a novel regulatory role for 14-3-3γ in Leydig cell steroidogenesis using the MA-10 cell line model (26). Steroidogenesis in MA-10 cells is triggered by human chorionic gonadotropin and its secondary messenger cAMP, leading to cholesterol transfer from cytosolic sources into mitochondria, which is the rate-limiting step in steroidogenesis. This import is mediated by a protein complex called the transduceosome that assembles in a hormone-dependent manner at the outer mitochondrial membrane (13). STAR is a hormone-induced protein found at the transduceosome where it likely acts to induce cholesterol transfer from the outer mitochondrial...
The goal of these protein/protein interactions and modifications is the creation of a permissive microenvironment that would allow maximal cholesterol delivery to cytochrome P450 11A1, and this steroid production peaks 120 min after hormone or cAMP treatment (28, 41). Although both the transducer and STAR are rapidly assembled at the outer mitochondrial membrane in response to hormone or cAMP treatment, maximal steroidogenesis occurs within 120 min, indicating a certain delay. Our previous study revealed that this delay is due to the interaction of cytosolic STAR with 14-3-3/H9253 (27, 42). Upon cAMP stimulation, 14-3-3/H9253 homodimers are disrupted, and the monomers interact with STAR on Ser194 in the 14-3-3 binding motif. Ser194 is a PKA phosphorylation site, which is responsible for maximal STAR activity for steroidogenesis. Mutation of this residue is linked with congenital adrenal lipoid hyperplasia due to cholesterol accumulation in the lipid droplets of adrenal cortex cells (26). 14-3-3/H9253 interactions with this site retain basal STAR activity in the cytosol. Dissociation of the two proteins is completed at 120 min, which coincides with high rates of 14-3-3y homodimerization, and leads to maximal induction of steroid production (29, 30). Therefore, the 14-3-3y cAMP- and time-dependent association and dissociation from STAR regulate the rate of steroidogenesis. As 14-3-3y homodimerization and STAR binding have opposite patterns, the results suggest a link between 14-3-3y homodimers and STAR binding (26).

The results of this study confirmed previous findings and further demonstrated that when the 14-3-3y target binding site is competed out using cell-penetrating peptides conjugated to this domain steroidogenesis is induced at time points when 14-3-3y and STAR interact. Conversely, when 14-3-3y dimerization is competed out, steroidogenesis is reduced at the time points when 14-3-3y homodimerizes and dissociates from STAR. Because 14-3-3y interacts with other 14-3-3 isoforms and together they target a variety of proteins, blocking dimerization or target-binding domains of 14-3-3y might not lead to a STAR-specific effect. Therefore, we searched for other
potential mechanisms regulating protein homodimerization that would not block the general dimerization or target-binding domains.

PTMs have important regulatory impacts on proteins and alter protein effects in different intracellular pathways. Phosphorylation of serine, threonine, and tyrosine residues and lysine acetylation are the most common PTMs (20). Dimerization and target protein modifications have been shown to be essential for 14-3-3 protein function (38), but recent studies demonstrated that 14-3-3 monomers can also bind target proteins as well as dimers, and 14-3-3 targets can be non- or dephosphorylated (31, 44). Moreover, high throughput screening using mass spectrometry identified 14-3-3 isoforms in phosphorylated or acetylated protein pools (2, 45–47). These PTMs occur on residues in dimerization and target-binding domains on the outer surface of 14-3-3 proteins, suggesting that these modifications affect 14-3-3 target binding or dimerization specificity. Modifications on Ser58 and Lys49 affected 14-3-3 protein self-dimerization and target binding, respectively, for the majority of 14-3-3 isoforms but not 14-3-3y (2, 13, 20).

We studied 14-3-3γ phosphorylation and acetylation during cAMP-induced steroidogenesis. The results indicated that phosphorylation and 14-3-3γ homodimerization display similar patterns as do 14-3-3γ acetylation and STAR interactions. Using TAT fusion peptides that compete endogenously with specific sequences on 14-3-3γ, we studied the impact of blocking such Lys49 and Ser58 modifications on STAR binding, 14-3-3γ homodimerization, and steroidogenesis in MA-10 cells. Phosphorylation of Ser58 and acetylation of Lys49 were shown to function in a coordinated and time-dependent manner in regulating 14-3-3γ function. Acetylation was shown to regulate the levels of 14-3-3γ dimers at 60 min post 8-Br-cAMP treatment, and phosphorylation regulated dimer levels throughout steroidogenesis, particularly at 120 min. Competition with either of these modifications significantly increased 14-3-3γ homodimer levels. Moreover, Ser58 phosphorylation levels seem to serve different purposes. First, although Ser58 phosphorylation was highest in 14-3-3γ homodimers, blocking this modification significantly increased homodimerization and therefore completely blocked 14-3-3γ/STAR interactions below basal levels, suggesting that maintenance of the levels of this PTM is critical for homodimerization. Despite the continuous induction in steroidogenesis under such conditions, the interactions between 14-3-3γ and STAR reach basal levels at 120 min when the 14-3-3γ negative regulatory role on STAR is terminated, whereas increased steroidogenesis is still observed. Such an induction suggests the presence of an alternate regulation on 14-3-3γ that remains to be identified. Moreover, a more moderate decrease in Ser58 phosphorylation was naturally observed in the cells in response to cAMP treatment. It is likely that subtle changes are responsible for 14-3-3γ functional regulation as they lead to a modest disruption of 14-3-3γ dimers, allowing the acetyl groups to occupy the proximal Lys49 and induce 14-3-3γ/STAR interactions. Acetylation and phosphorylation therefore act inversely as observed at 120 min, and acetylation is replaced by phosphorylation, and the two proteins dissociate. Lys49 acetylation is essential for the 8-Br-cAMP-dependent induction of 14-3-3γ/STAR. If this PTM is blocked, 14-3-3γ/STAR interactions remain at basal levels, and 14-3-3γ homodimerization is subsequently induced at 30–60 min. Steroidogenesis is therefore induced more intensely at 60 min. Although the function of the identified Ser58 and Lys49 PTM sites is clear, the role of other PTM sites that may contribute to 14-3-3γ protein function cannot be ruled out.

Interestingly, free cholesterol and cholesterol storage levels in MA-10 cells were reduced when Lys49 acetylation or Ser58 phosphorylation of 14-3-3γ was blocked. 8-Br-cAMP treatment is in part responsible for the increased use of stored and free cholesterol for steroidogenesis. However, the dramatic reduction of lipid droplets in response to the treatment with TAT-14-3-3γ Lys49 and TAT-14-3-3γ Ser58 (much beyond the activity of 8-Br-cAMP) suggests some additional function. Miller and co-workers reported that mutations on the Ser residues, such as Ser194 on STAR, can inhibit STAR activity, and therefore patients with such mutations were diagnosed with congenital adrenal lipoid hyperplasia, a condition in which steroidogenesis is impaired and lipids accumulate in the adrenal cortex (48). However, upon addition of TAT-14-3-3γ Lys49 or TAT-14-3-3γ Ser58, there was a significant reduction in the MA-10 cell lipid pool, indicating that if modifications of 14-3-3γ are competed out by these fusion peptides 14-3-3γ interactions with STAR are consequently reduced, resulting in free cytosolic cholesterol and depleting lipid droplets of cholesterol, cholesterol esters, and likely other lipids (49) as steroidogenesis increases. It would be of interest to examine whether TAT-14-3-3γ Ser58 or TAT-14-3-3γ Lys49 can reverse the symptoms of lipoidal congenital adrenal lipid hyperplasia. These findings highlight the significant role of 14-3-3γ in the regulation of acute steroidogenesis to avoid uncontrolled lipid-to-steroid conversion. Moreover, because of the high homology of 14-3-3γ proteins, we studied the potential of the peptides used to compete out PTMs on 14-3-3α, the isoform in this family that is involved in steroidogenesis. 14-3-3α contains an alanine instead of Ser58 and therefore is unaffected by the TAT-14-3-3α Ser58 peptide. However, 14-3-3ε contains Lys49. Although the effect of TAT-14-3-3α Lys49 is observed before the activation of the ε isoform in steroidogenesis, at present we cannot rule out the possibility that over longer periods of time treatment the TAT-14-3-3α Ser58 fusion peptide may also act on 14-3-3ε.

A simplified scheme is proposed as a model for 14-3-3γ PTMs, which regulate steroidogenesis through interactions with STAR (Fig. 7). At basal levels, 14-3-3γ is highly phosphorylated and therefore present as homodimers. Under these conditions, STAR levels in MA-10 cells are low. 8-Br-cAMP treatment for 30 min triggers steroidogenesis, reducing phosphorylation of 14-3-3γ monomers on Ser58, leading to disruption of 14-3-3γ dimers (26). CAMP treatment for 60 min induces 14-3-3γ and STAR expression (22) and leads to high acetylation of 14-3-3γ on Lys49. This modification disrupts the 14-3-3γ dimerization surface; hence, 14-3-3γ dimer levels are reduced, whereas 14-3-3γ/STAR interactions are induced, blocking STAR activation. If this modification is blocked, the interactions between the two proteins remain at the basal level. After 120 min of cAMP treatment, acetylation is
**14-3-3γ Modifications Regulate Its Steroidogenic Role**

![Diagram of 14-3-3 protein complexes at different time points: Basal, cAMP 30', cAMP 60', cAMP 120']

**FIGURE 7. Schematic representation of 14-3-3γ PTM steroidogenesis regulation.** At basal levels, 14-3-3γ is highly phosphorylated on Ser58 (shown by two phosphate (P) groups). cAMP stimulation for 30–60 min (’) reduces Ser58 phosphorylation and increases Lys49 acetylation (indicated by A), inducing the 14-3-3γ/STAR interaction. After 120 min of cAMP treatment, 14-3-3γ phosphorylation is recovered, and acetylation is decreased; therefore, homodimerization of 14-3-3γ is triggered, and STAR is released from 14-3-3γ. The Ser195 STAR phosphorylation site is accessible to PKA, inducing its function and triggering maximal steroidogenesis. TSPO, translocator protein; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

reduced, and phosphorylation levels are increased, leading to homodimerization of 14-3-3γ and disruption of STAR binding, allowing PKA to phosphorylate Ser194 on STAR to induce dimerization, although PTMs are shown for Ser58 but not the blocked. This indicates that both sites regulate protein satory mechanism. Indeed, blocking the 14-3-3 like that observed when the Ser195 STAR phosphorylation site is triggered, and STAR is released from 14-3-3γ. The Ser195 STAR phosphorylation site is accessible to PKA, inducing its function and triggering maximal steroidogenesis. TSPO, translocator protein; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

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