Phosphorylation does not regulate the activity of steroidogenic acute regulatory protein (STAR): Disputing the STAR phosphoprotein narrative

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The Steroidogenic Acute Regulatory (STAR) protein is a cholesterol shuttle in the mitochondrial inter-membrane space crucial for all *de novo* steroidogenesis. In demonstrating this mechanism of action in recent studies, we were puzzled how a protein that undergoes co-translational import and ultimately folds in the mitochondrial intermembrane space can encounter phosphorylation by the cytoplasmic enzymes: cAMP-dependent protein kinase (PKA) or the mitogen-activated protein kinases (MAPKs), that have been implicated in what is considered a key regulatory step in STAR function.

In prior literature, points of STAR phosphorylation were determined based on conserved consensus sites to be the Serine (Ser) amino acids: Ser55, Ser56, and Ser194. Functional assessments for each of these possibilities were carried out in an induced- steroidogenic system using COS-1 cells by expressing STAR mutants that replaced Ser with Alanine (Ala). From these experiments it was suggested that mutations eliminating the ability of STAR phosphorylation at Ser194Ala resulted in hypomorphic STAR with reduced steroidogenic function. Mutations at Ser55Ala and Ser56Ala had no effects. In analyzing this literature carefully, we identified that although Ala lacks a side-chain that can be phosphorylated, it is a hydrophobic amino acid (compared to the hydrophilic Ser that it replaces), a shift could disrupt the protein's structure/function. Therefore, we decided to reexamine these mutations by replacing the Ser with another hydrophilic amino acid, Glycine (Gly), that would not only lack a side-chain for phosphorylation but also avoid any inadvertent disruptions to the STAR secondary structure.

For our experiments, we used MA10 Leydig STAR knockout cells ($MA10^{STKO}$) for precisely testing functional recovery after reconstituting expression of wildtype STAR or different STAR mutants constructed in lentiviral vectors using precise site-directed mutagenesis. Our findings showed that all the STAR mutants (Ser55Gly, Ser56Gly and Ser194Gly) were able to fully support steroidogenesis in $MA10^{STKO}$ cells. This raised the question whether previous conclusions that were purported to indicate phosphorylation with Ser194 were in fact indicative of a structural/functional interference caused by Ala mutation.

These findings allow us to conclude that the premise of STAR being regulated by phosphorylation is unsubstantiated. This is reinforced by the fact that there is no direct mass spectrometry evidence for a STAR phosphoprotein. Previous work on ^{32}P incorporation in expressed STAR was performed using COS1 cells co-expressing a designer protein fusion (termed F2) that integrated CYP11A1, adrenodoxin and adrenodoxin reductase, a system that has supported cytoplasmic STAR activity without mitochondrial targeting. Our results also challenge the signaling assumptions that PKA and MAPKs can directly target and regulate STAR, thereby highlighting the need to reevaluate upstream signaling mechanisms relevant for the acute regulation of steroidogenesis.