Phosphorylation of Steroidogenic Acute Regulatory Protein (StAR) Modulates Its Steroidogenic Activity*

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Steroidogenic acute regulatory protein (StAR) plays a critical role in steroid hormone synthesis. StAR is thought to increase the delivery of cholesterol to the inner mitochondrial membrane where P450sec resides. Tropic hormones acting through the intermediacy of cAMP rapidly increase pregnenolone synthesis, and this rapid steroidogenic response is believed to be due to StAR's action. The StAR protein contains two consensus sequences for phosphorylation catalyzed by protein kinase A that are conserved across all species in which the amino acid sequence of the StAR protein has been determined. We demonstrated that human StAR expressed in COS-1 cells exists in at least four species detectable by two-dimensional gel electrophoresis followed by Western blotting. The two more acidic species disappeared after treatment of the cell extracts with alkaline phosphatase. 32P was incorporated into StAR protein immunoprecipitated from COS-1 cell extracts, and a 10-min treatment with 8-bromo-cAMP increased 32P incorporation by in vitro transcription/translation. StAR protein generated by in vitro transcription/translation was phosphorylated by the protein kinase A catalytic subunit in the presence of [γ-32P]ATP. Mutation of potential sites for protein kinase A-mediated phosphorylation at serine 57 and serine 195 to alanines, individually, reduced 32P incorporation from labeled ATP into StAR preprotein produced by in vitro transcription/translation when incubated with protein kinase A catalytic subunit. 32P labeling of StAR protein expressed in COS-1 cells was also reduced when serine 57 or serine 195 were mutated to alanines. A double mutant in which both serine 57 and serine 195 were changed to alanines displayed markedly reduced 32P incorporation. To determine the functional significance of StAR phosphorylation, we tested the steroidogenic activity of the wild-type StAR and mutated StAR proteins in COS-1 cells expressing the human cholesterol side chain cleavage enzyme system. Mutation of the conserved protein kinase A phosphorylation site at serine 57 had no effect on pregnenolone synthesis. However, mutation of the serine residue at 195 resulted in an approximately 50% reduction in pregnenolone production. The S195A mutant construct did not yield the more acidic species of StAR detected in two-dimensional Western blots, indicating that the mutation affected the ability of the protein to be post-translationally modified. Mutation of the corresponding serine residues in murine StAR (Ser56 and Ser194) to alanines yielded results that were similar to those obtained with human StAR; the S56A mutant displayed a modest reduction in steroidogenic activity, whereas the S194A mutant had approximately 40% of the activity of murine wild-type StAR. In contrast to the human S195A mutation, conversion of serine 195 to an aspartic acid residue had no effect on steroidogenic activity, consistent with the idea that a negative charge at this site modulates StAR function. Our observations suggest that phosphorylation of serine 194/195 increases the biological activity of StAR and that this post- or co-translational event accounts, in part, for the immediate effects of cAMP on steroid production.

The recently discovered steroidogenic acute regulatory protein (StAR) plays a critical role in the initial steps in steroidogenesis (1). Mutations in the StAR gene that inactivate the protein cause the most severe form of congenital adrenal hyperplasia in which the synthesis of all gonadal and adrenal steroids is markedly impaired (2, 3). The StAR protein was originally identified by Orme-Johnson and colleagues (4–7) and Stocco and associates (8–11) as a 30-kDa phosphoprotein associated with mitochondria in gonadal and adrenal cells. Studies by Orme-Johnson and coworkers (5) first demonstrated a direct correlation between the appearance of a phosphorylated form of StAR and steroidogenesis. These authors suggested that StAR undergoes co-translational phosphorylation in response to cAMP, generating the active form of the protein.

The cDNAs encoding StAR have been determined for mouse (11), human (12), hamster (13), rat (14), cow (15), sheep (16), and pig (17). Based on the comparison of the deduced amino acid sequences from these species and the predicted consensus motifs for protein kinase A (PKA) phosphorylation (R-R/K-S/T) (18), two conserved putative sites for PKA phosphorylation were identified (Fig. 1). These sites are serine 56/57 and serine 194/195 in the murine and human sequences, respectively. In the present study, we examined the roles of the serine 56/57 and serine 194/195 residues in StAR's steroidogenic activity.

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The abbreviations used are: StAR, steroidogenic acute regulatory protein; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; 8-BrcAMP, 8-bromo-cyclic AMP.

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2 J. L. Juengel, B. M. Meberg, and G. D. Niewender, personal communication.
**Phosphorylation of StAR**

**TECHNOLOGIES, INC.) with 1.0**

SPORT-1 with 1.0 plasmid, the wild-type, or mutant human StAR cDNAs in pSV-

**after 24 h, and some cultures received 5**

pCMV-5-based plasmids were used. The culture media were changed (1, 3, 20), kindly provided by Dr. Walter L. Miller, University of Cali-

**fuged at 600**

studies of human StAR expression. The anti-murine StAR was used for

**peptide sequence in murine StAR using previously published methods**

protein, to be described elsewhere (32), or antibody raised against a

**multiple range test was used to evaluate differences between control**

ments were repeated on at least three separate occasions. Duncan's

**maximal cholesterol side chain cleavage activity (3, 19). Each experi-

**formation in the presence of the exogenous substrate, which reflects**

production in the absence of 22( hydroxycholesterol to pregnenolone

**Dishes in homogenization buffer consisting of 0.25M sucrose, 10 m M**

**Tris-HCl, pH 7.4, 10 m M EDTA, and 5**

**32P and 35S and Immunoprecipitation of StAR Pro-

**tein—The COS-1 cells transfected with plasmid directly expressing**

**wild-type and mutant StAR proteins were rinsed and incubated in**

**phosphate-free Dulbecco's modified Eagle's medium without serum for**

**20 min and then labeled with [32P]orthophosphate (200**

**Ci/ml). After a 30-min incubation at 30 °C, the reaction was stopped by**

**addition of SDS sample buffer (22) and then subjected to SDS-PAGE and**

auradiorography.

**In Vitro Phosphorylation of StAR Proteins by PEA—Wild-type and**

**mutant StAR proteins were synthesized, using an SP6 T7-coupled**

**in vitro** transcription/translation kit following the manufacturer's** protocol (Promega) for 2 h at 30 °C. The **in vitro** translated proteins were incubated with 10 units of protein kinase A (PKA) catalytic subunit (Promega) in 10 mM HEPES buffer, pH 7.5, 10 mM MgCl$_2$, 1 mM EGTA, and 0.1 mM y-32P]ATP in a total volume of 50 μl. After a 30-min incubation at 30 °C, the samples were resolved by SDS-PAGE (22) followed by autoradiography as described above.

**RESULTS**

**Human StAR Is Phosphorylated—Two-dimensional PAGE** followed by Western blotting of extracts of COS-1 cells transfected with a human StAR expression plasmid demonstrated the presence of immunoreactive 30-kDa proteins, representing mature StAR, in four distinct forms (Fig. 2A). Treatment of the COS-1 cell extracts with alkaline phosphatase prior to electro-

**horesis revealed that the more acidic immunoreactive pro-

**teins were sensitive to phosphatase treatment, suggesting that**

**they are phosphoproteins (Fig. 2B). Since phosphatase treat-

**ment collapsed the four major immunoreactive proteins into**

**two major spots, it appears that some other post-translational**

**modification of StAR or phosphatase-insensitive modification**

**accounts for the presence of at least one of the species. This**

**finding is consistent with the earlier reports of Orme-Johnson**

**and colleagues (4–7) and Stocco and co-workers (8–11). The**

**presence of phosphorylated forms of StAR in the transfected**

**COS-1 cells suggests that these hosts have the ability to gen-

**erate these species in the basal state.**

**COS-1 cells expressing wild-type StAR were labeled with**

**[32P]orthophosphate overnight, and some cultures were treated with**

**1 mM 8-Br-cAMP for 10 min. Autoradiography performed on**

**immunoprecipitated StAR proteins resolved by SDS-PAGE revealed**

**incorporation of [32P] into both pre- and mature StAR pro-

**teins (Fig. 3). The labeling of StAR preprotein was signifi-

**cantly increased by the treatment with 8-Br-cAMP and the**

**mobility of the preprotein from the cAMP-treated cell ex-

**tracts was slightly retarded compared with the StAR prepro-

**tein in untreated cells. In addition, radiolabeled bands inter-

**mediate between the preprotein and 30-kDa mature protein**

**were observed in the immunoprecipitates from 8-Br-cAMP-**

**treated cells. This suggests sequential processing of the StAR**

**using site-directed mutagenesis of these potential sites of**

**cAMP-stimulated phosphorylation.**

**MATERIALS AND METHODS**

**Construction of Mutant StAR cDNA Constructs—The S7A, S195A,**

**S195D, and S7A/S195A mutations were produced by site-directed**

**mutagenesis using reagents purchased from CLONTECH. The wild-

**type and mutant human StAR cDNAs were cloned into pSV-SPORT-1.**

**Each construct was sequenced to confirm the mutation(s) as described**

**previously (19). The murine StAR cDNA constructs were prepared by**

**Genosys (The Woodlands, TX). They were subcloned into the pCMV-5**

**eukaryotic expression vector.**

**Cell Culture and Evaluation of Steroidogenic Activity—COS-1 cells**

**were cultured in 6- or 12-well plastic culture plates to 50–80% conflu-

**ence. The cells were transfected using 10 μg/ml LipofectAMINE (Life**

**Technologies, Inc.) with 1.0 μg/ml of either an empty pSV-SPORT-1**

**plasmid, the wild-type, or mutant human StAR cDNAs in pSV-

**SPORT-1 with 1.0 μg/ml of a plasmid-directing expression of a fusion**

**protein consisting of P450scc, adrenodoxin and adrenodoxin reduc-

**tase (1, 3, 20), kindly provided by Dr. Walter L. Miller, University of Cali-

**fornia, San Francisco. In studies of murine StAR, the corresponding**

**pCMV-5-based plasmids were used. The culture media were changed**

**after 24 h, and some cultures received 5 μg/ml 22R-hydroxycholes-

**terol. At the end of the treatment period, the media were collected for**

**evaluation of steroidogenic activity, and the cells were scraped from the**

**dishes in homogenization buffer consisting of 0.25 m sucrose, 10 mM**

**Tris-HCl, pH 7.4, 10 mM EDTA, and 5 μg/ml aprotinin (19). Relative**

**steroidogenic activity was determined by normalizing pregnenolone**

**production in the absence of 22( hydroxycholesterol to pregnenolone**

**formation in the presence of the exogenous substrate, which reflects**

**maximal cholesterol side chain cleavage activity (3, 19). Each experi-

**ment included triplicate cultures for each treatment group. Experi-

**ments were repeated on at least three separate occasions. Duncan's**

**multiple range test was used to evaluate differences between control**

**and mutated proteins.**

**Western Blotting—COS-1 cell extracts were harvested for Western**

**blot analysis with an antibody raised against recombinant human**

**StAR protein, to be described elsewhere (32), or antibody raised against a**

**peptide sequence in murine StAR using previously published methods**

**(11, 19). The anti-recombinant human StAR antibody was used in**

**studies of human StAR expression. The anti-murine StAR was used for**

**studies of murine StAR expression. COS-1 cells collected into homo-

**genization buffer were sonicated for 5 s. The disrupted cells were centri-

**fuged at 600 × g for 15 min and the resulting supernatant was used for**

**the Western blot analysis as described previously (19).**

**Two-dimensional Gel Electrophoresis—Equal amount of protein (10 μg)**

**from each sample were loaded onto isoelectric focusing gels in**

**capillary tubes prepared with ampholytes with a pH range of 3 to 10.**

**Two-dimensional SDS-PAGE was performed as described previously**

**(21). The pH gradients of the isoelectric focusing gels were modified**

**using two-dimensional standards obtained from Bio-Rad. After elec-

**trophoresis, the gels were transferred to nitrocellulose membranes for**

**probing with anti-StAR antibody. In some cases, samples were pre-

**treated with 1 IU of intestinal alkaline phosphatase (Boehringer Mann-

**heim) for 30 min at 37 °C prior to two-dimensional gel electrophoresis.**

**Incorporation of [32P] and [35S] and Immunoprecipitation of StAR Pro-

**tein—**

**A**

| Bovine | QVR | RRS | LLG | Bovine | KRGS | MCVLA |
|--------|-----|-----|-----|--------|------|-------|
| Hamster | QVR | RRS | LLG | Hamster | KRGS | T CVLA |
| Human | QVR | RRS | LLG | Human | KRGS | T CVLA |
| Mouse | QVR | RRS | LLG | Mouse | KRGS | T CVLA |
| Porcine | QVR | RRS | LLG | Ovine | KRGS | MCVLA |
| Rat | QVR | RRS | LLG | Porcine | KRGS | VC MLA |
| Consensus | QVR | RRS | LLG | Rat | KRGS | T CVLA |

**FIG. 1. StAR contains two conserved consensus PKA phosphory-

**lation sites, Panels A (serine 56/57) and B (serine 194/195) show**

**aligned deduced amino acid sequences of StAR proteins from the indi-

**cated species. Amino acid residues differ among species due to the absence of one residue in the amino terminus in hamster, murine, and rat StAR. The conserved motif with target**

**serine residue in panel A is also observed in ovine StAR (J. L. Juengel,**

**B. M. Meberg, and G. D. Niswender, personal communication). Boxes**

**denote consensus PKA phosphorylation motifs.**
preproteins in the absence of added PKA catalytic subunit generate the wild-type and mutant proteins (Fig. 4). We also transfected COS-1 cells with the wild-type and mutated StAR expression plasmids to compare the phosphorylation of mutant StAR proteins in the context of intact cells. After the COS-1 cells were labeled with [32P]orthophosphate, they were stimulated with 1 mM 8-Br-cAMP for 10 min, and then cellular extracts were subjected to immunoprecipitation, SDS-PAGE, and autoradiography (Fig. 5). The incorporation of 32P into the wild-type StAR preprotein was increased by 8-Br-cAMP treatment. The cAMP analog also provoked some increase in 32P labeling of the S57A and S195A mutants, but not the double S57A/S195A mutant. cAMP treatment also retarded the mobility of the wild-type StAR preprotein, as noted above. In addition, 32P incorporation into the mature StAR proteins was found in cells transfected with wild-type and S57A mutant, but not S195A and S57A/S195A double mutants.

When extracts of COS-1 cells transfected with the S195A StAR mutant were subjected to two-dimensional Western blot analysis, the pattern of immunoreactive proteins resembled that of the alkaline phosphatase-treated extracts of wild-type COS-1 cells and the intermediate proteins between the pre- and mature StAR proteins (Fig. 3). The mobility of the StAR preprotein, as noted above. In addition, 32P incorporation into the mature StAR proteins was found in cells transfected with wild-type and S57A mutant, but not S195A and S57A/S195A double mutants.

Steroidogenic Activity of StAR Phosphorylation Mutants—To examine the functional importance of phosphorylation of serine 57 and serine 195, we tested the steroidogenic activity of mutant proteins in which these serine residues were converted to alanines using COS-1 cells co-transfected with the cholesterol
Fig. 4. Phosphorylation of StAR preproteins produced by in vitro transcription/translation by PKA. A, Western blot showing the expression of the in vitro translated wild-type (WT) and mutant StAR proteins. B, in vitro translated wild-type StAR preproteins were incubated with [γ-32P]ATP in the absence (−) or presence (+) of PKA catalytic subunit. C, in vitro translated wild-type and mutant StAR preproteins were incubated with [γ-32P]ATP and PKA catalytic subunit. [35S]Methionine/cysteine-labeled wild-type StAR preproteins produced by in vitro transcription/translation were analyzed as standards on the same gel (control [35S]).

Fig. 5. Phosphorylation of wild-type and mutant StAR proteins in COS-1 cells. COS-1 cells transfected with wild-type and the indicated mutant StAR expression plasmids were labeled with 200 μCi/ml [35S]orthophosphate for 5 h. Some cultures were treated with 1 mM 8-Br-cAMP for 10 min. StAR proteins were immunoprecipitated from the COS-1 cell extracts and resolved by SDS-PAGE, and the labeled proteins were visualized by autoradiography. Arrow, StAR preprotein in unstimulated COS-1 cells; filled arrowhead, StAR preprotein in 8-Br-cAMP-treated COS-1 cells; scalloped arrowheads, intermediate StAR forms; open arrowhead, mature StAR protein.

Discussion

Our observations demonstrate that human StAR protein is phosphorylated on serine residues at codons 57 and 195. These residues are in the context of consensus PKA phosphorylation sites, and these consensus sequences are conserved in the StAR proteins of all species studied to date. We have provided evidence that the phosphorylation of residues serine 57 and serine 195 is catalyzed by PKA and that incorporation of phosphate into these residues is increased by cAMP treatment of cells. However, it should be noted that the StAR protein may be phosphorylated at other sites and by other protein kinases including calcium/calmodulin-dependent protein kinase II (23), which phosphorylates serine or threonine residues in a context similar to that of PKA (18) and protein kinase C (7). StAR does not appear to be phosphorylated on tyrosine residues since we have not demonstrated a reaction of human or murine StAR with anti-phosphotyrosine antibodies.3

Mutation of the serine at codon 195 in human StAR and the serine at codon 194 in murine StAR to nonphosphorylatable alanine residues reduces StAR’s steroidogenic activity by approximately 50%, while mutation of serine 195 in human StAR to an aspartic acid residue slightly increases relative steroidogenic activity. Collectively, this evidence suggests that phosphorylation of serine 194/195 modulates StAR’s functional activity. It is unlikely that the conversion of the serine 194/195 to an alanine diminished StAR’s activity as a consequence of protein misfolding. First, the levels of expression of the S194A/S195A mutants were not significantly different from wild-type murine or human StAR or the S195D human StAR mutant. Second, the S195A mutant was labeled and processed to the mature protein by PKA, and mutation of serine 195 in human StAR to an alanine residue reduced StAR’s steroidogenic activity by approximately 50%, while mutation of serine 195 in human StAR to an aspartic acid residue slightly increases relative steroidogenic activity. Collectively, this evidence suggests that phosphorylation of serine 194/195 modulates StAR’s functional activity. It is unlikely that the conversion of the serine 194/195 to an alanine diminished StAR’s activity as a consequence of protein misfolding. First, the levels of expression of the S194A/S195A mutants were not significantly different from wild-type murine or human StAR or the S195D human StAR mutant. Second, the S195A mutant was labeled and processed to the mature protein similarly to wild-type StAR. Third, we have previously reported that mutation of the adjacent threonine at codon 196 in human StAR to an alanine does not affect steroidogenic activity, nor does mutation of serine 100 or serine 277 to alanines (24).

3 F. Arakane, S. R. King, Y. Du, C. B. Kallen, L. P. Walsh, H. Watari, D. M. Stocco, and J. F. Strauss, III, unpublished observations.
S195A mutant StAR protein is localized to mitochondria by immunohistochemistry, just like wild-type StAR, and the S195A preprotein is as efficiently transported into isolated mitochondria and processed to mature protein in \textit{in vitro} import assays as wild-type StAR preprotein.\footnote{F. Arakane and J. F. Strauss, III, unpublished observations.} The fact that the S56A/S57A mutation failed to significantly affect the steroidogenesis enhancing activity of the protein is not unexpected given our past observation that the first 62 amino-terminal residues of human StAR can be deleted without influencing steroidogenic activity (19). Because the S194A/S195A mutants retain 50\% of the steroidogenic activity of wild-type StAR, we must conclude that in the context of the transfected cell system we used, phosphorylation of StAR at serine 194/195 is not absolutely required for the protein’s function.

\textbf{Fig. 6. Relative steroidogenic activity of wild-type and phosphorylation site StAR mutants.} A, COS-1 cells were transfected with the indicated human StAR plasmid and an expression plasmid for the human cholesterol side chain cleavage enzyme system. Relative steroidogenic activity assayed as pregnenolone production normalized to the conversion of 22(\(R\))-hydroxycholesterol to pregnenolone is presented, taking the wild-type StAR value as 100\%. Values are means \(\pm\) S.E. from four separate experiments. B, Western blot analysis was carried out on extracts of COS-1 cells to demonstrate expression of the human wild-type and mutant StAR proteins using antirecombinant human StAR antiserum. C, effects of mutation of serine residues 56 and 194 in murine StAR on pregnenolone synthesis. COS-1 cells were transfected with expression plasmids for murine wild-type StAR or the S56A or S194A mutants and pregnenolone secretion was determined. The results of a representative experiment replicated five times is presented. Values are presented with standard deviations. D, Western blot analysis of murine StAR proteins expressed in COS-1 cells using the anti-peptide antibody to murine StAR sequences.
Our findings are consistent with the notion that the activity of StAR can be increased by a co- or post-translational modification as originally suggested by Orme-Johnson and colleagues (5). StAR may be phosphorylated at serine residue 194/195 in response to an acute increase in cAMP levels and the subsequent activation of PKA. Since StAR has a short half-life, any change in the biological activity of newly synthesized StAR, as might result from phosphorylation of serine 194/195, would have a significant but short-lived effect on steroidogenesis. Thus, phosphorylation of StAR may be part of the mechanism of the immediate increase in steroid production following tropic stimulation of the adrenal cortex and gonads by ACTH and luteinizing hormone, respectively. cAMP also has long term effects on steroidogenesis by increasing the abundance of StAR mRNA and StAR protein, primarily through increases in the rate of transcription of the StAR gene (25, 26). The exact mechanism by which phosphorylation changes the steroidogenic activity of StAR will remain a matter of speculation until the fundamental process by which StAR stimulates pregnenolone synthesis and the pathways that lead to its inactivation are elucidated.

Our two-dimensional Western blot studies indicate that COS-1 cells process StAR in a fashion that is similar to that previously reported in animal steroidogenic cells (4–10). Several distinct charge isofoms were found and the more acidic species were sensitive to alkaline phosphatase treatment, consistent with the idea that they are derived from phosphorylation of the more basic forms of the protein. Despite this similarity, the COS-1 cell system has shortcomings that limit its utility for the study of the acute effects of cAMP on StAR function. Unlike normal steroidogenic cells, COS-1 cells co-transfected with the cholesterol side chain cleavage system expression plasmid used in these studies and Drs. Charles Strott (National Institutes of Health) and Focko F. G. Rommerts (Erasmus University, Rotterdam, The Netherlands) for providing anti-pregnenolone antibodies used in the pregnenolone radioimmunosassays.

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