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REVIEW

The Steroidogenic Acute Regulatory (StAR) Protein Two Years Later

An Update

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Scope of Review

It has now been approx 2 yr since the purification, cloning, sequencing, and expression of the Steroidogenic Acute Regulatory (StAR) protein. This article will attempt to not only catalog the earlier studies that have been performed on this steroidogenic tissue-specific, trophic hormone-induced protein, but also to update what has recently been found. The intention of this article is, therefore, to summarize the available data and then integrate this data into what is thought to be its role in the acute regulation of steroid hormone biosynthesis. A number of review articles and commentaries on this subject have also recently appeared *(1–8)*. In several of those articles, a much wider scope of the field of the acute regulation of steroidogenesis was included. Many of those observations will not again be presented here. Rather, the subject of this review will be confined to what has been learned in the past and what is currently known about the StAR protein. As in any ongoing scientific endeavour, much of which has been observed, and which will be described herein, is fact and much of that which will be discussed, especially with regard to the mechanism of action of StAR, is more speculative.

Background

The steroid hormones consist of compounds that are synthesized in the adrenal gland, the ovary, the placenta, and the testis. There is also an additional class of steroids known as the neuroactive or neural steroids that are synthesized by the central nervous system (CNS) and appear to have specialized functions in this tissue *(9)*. A common characteristic shared by all the steroid hormones, regardless of the tissue of origin, is that they are synthesized from a common substrate, cholesterol, and the biosynthesis of all steroids begins with the enzymatic cleavage of the side chain of cholesterol to form the first steroid synthesized, pregnenolone. This reaction is catalyzed by the cytochrome P450 side chain cleavage enzyme (P450*scc*), which is part of the cholesterol side chain cleavage enzyme system (CSCC), and which is located on the matrix side of the inner mitochondrial membrane *(10–13)*. At this point in time, it is clear that in order to initiate and sustain steroidogenesis, a constant supply of the substrate cholesterol must be readily available within the cell for transport to the site of its cleavage in the inner mitochondrial membrane. Once sequestered within the steroidogenic cell, cholesterol transport in response to trophic hormone stimulation occurs in two separate processes. The first phase is the mobilization of cholesterol from cellular stores such as lipid droplets or plasma membranes to the outer mitochondrial membrane, whereas the second part consists of the transfer of cholesterol from the outer to the inner mitochondrial membrane *(14)*. The factors and processes that are responsible for the mobilization of cholesterol to the outer mitochondrial membrane are not well understood, but are clearly important considerations when analyzing the events involved in hormone-regulated steroidogenesis, and have been the subject of intensive investigation *(15–22)*.

Whereas much of the early work that was performed on the steroidogenic pathway indicated that the acute rate-limiting step in this process was the hormone-induced increase in the activity of the P450*scc*, which functioned to convert cholesterol to pregnenolone *(23–25)*, this is, in fact, not the case. A number of observations by several different investigators indicated that the true rate-limiting step affected by hormone stimulation in this process was the delivery of the substrate, cholesterol, from the outer to the inner mitochondrial membrane and the P450*scc* enzyme *(24,26–34)*. As proof of this, it was demonstrated that when more hydrophilic hydroxylated analogs of cholesterol such as 22R-hydroxycholesterol, 20α-hydroxycholesterol or 25-hydroxycholesterol, all of which can readily diffuse to the P450*scc*, are placed on steroidogenic cells, high levels of steroids could be produced without hormone stimulation *(35–38)*. These observations indicated that the P450*scc* was fully active and that it was the lack of cholesterol for cleav-

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age that prevented the production of pregnenolone and subsequent downstream steroids. Since the diffusion of the hydrophobic cholesterol through the aqueous layer between the outer and inner mitochondrial membrane is extremely slow *(17,19,22)*, it became clear that the transfer of cholesterol from the outer membrane to the P450*scc* must occur in an assisted manner and that this assisted transfer was the rate-limiting step. Therefore, in simple terms, the overall production of steroidogenesis is controlled by factors that facilitate the transport of cholesterol from lipid droplets and other cellular stores to the mitochondrial outer membrane and its subsequent translocation across the aqueous intermembrane space of the mitochondria to the inner membrane. The latter event is the key hormone-stimulated acute regulatory step.

The Role of Protein Synthesis

Because of the fundamental importance of the steroid hormones, there necessarily arose a desire to better understand the nature of their regulation. It quickly became apparent that steroid production in response to hormone stimulation had an absolute requirement for the synthesis of new proteins. The first of such studies were performed by Ferguson who demonstrated that the acute stimulation of corticoid synthesis in adrenal glands by adrenocorticotrophic hormone (ACTH) was sensitive to the protein synthesis inhibitor puromycin *(39,40)*. At approximately this same time, Garren and coworkers conducted a series of studies that clearly demonstrated that steroidogenesis in adrenal tissue was highly dependent upon the synthesis of new proteins in response to ACTH treatment *(41–44)*. An additional and most interesting observation was also made by Garren and coworkers who performed experiments on the capacity of prestimulated adrenal cells to continue steroid production in the presence of added cycloheximide. These studies concluded that the half-life of the putative stimulating factor was very short, thus giving rise to yet another descriptive phrase for the factor, "highly labile" *(41)*. Following these early observations, there were many similar studies performed that were all confirmatory of the need for *de novo* protein synthesis in the hormone regulated acute production of steroids *(24,28,45–56)*. Studies by van der Molen and colleagues also confirmed the need for *de novo* protein synthesis in the stimulation of steroid production in rat Leydig cells and even identified two possible protein candidates *(57,58)*. Importantly, Simpson et al. *(10)* determined that the cycloheximide sensitive step in this process was located in the mitochondria, but, just as importantly, it was also noted in a separate study that protein synthesis inhibitors had no effect on the activity of the P450*scc* itself *(59)*. It was also observed that inhibition of protein synthesis had no effect on the increased delivery of cellular cholesterol to the mitochondria, but that the delivery of this substrate from the outer mitochondrial membrane to the inner mitochondrial membrane was completely inhibited by cycloheximide *(28,60)*. Thus, the precise site of the cycloheximide inhibited regulation had been located. The observation that *de novo* protein synthesis was indispensable for the acute production of steroids in response to hormone stimulation has also been made more recently in several different steroidogenic tissues *(61–65)*. These observations have also been demonstrated in both mouse Y-1 adrenal tumor cells and MA-10 mouse Leydig tumor cells in which it was demonstrated that cholesterol could be delivered to a "presteroidogenic pool" in the presence of cycloheximide *(56)*. However, pregnenolone production did not take place until the inhibitor was removed and the cells subsequently stimulated with hormone. As a result of these many observations, it was stated that the acute production of steroids was dependent upon a hormone-stimulated, rapidly synthesized, cycloheximide-sensitive, and highly labile protein whose function appeared to be to transfer cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane and the P450*scc* enzyme.

The Steroidogenic Acute Regulatory (StAR) Protein

As stated earlier, whereas there have been a number of candidates put forth as the acute regulator of steroid hormone biosynthesis (as reviewed in ref. *3*), they will not all be discussed in this review. Rather, the protein candidate StAR, will be the focus of this work.

Proteins similar to, or identical to StAR were first described in a carefully conducted series of experiments by Orme-Johnson and colleagues as ACTH-induced 30 kDa proteins and phosphoproteins in rat adrenocortical cell suspensions *(61,66)*. The synthesis of this unique protein, termed i_b, was shown to occur with similar kinetics and dose response as corticosterone production following trophic hormone stimulation. Temporal correlations between the appearance of i_b, (now termed pp30), and steroid production were confirmed in additional steroidogenic cells, namely rat corpus luteum cell cultures and mouse adrenal and Leydig cell cultures *(67,68)*. The studies from Orme-Johnson's laboratory demonstrated that synthesis of the pp30 protein was sensitive to cycloheximide, but when cycloheximide was added to adrenal cells after steroidogenesis was stimulated by ACTH, the pp30 protein did not disappear quickly, whereas the decline in steroidogenesis occurred with a half-life of approx 5 min. This apparent discrepancy was solved when it was determined that pp30 was a mitochondrial protein and cytosolic precursors of this protein having molecular weights of approx 37 kDa and 32 kDa were identified *(62,69,70)*. When Epstein and Orme-Johnson determined that there existed a precursor–product relationship between the 37, 32, and 30 kDa proteins and that the half-life of the precursors were very short, a potential answer to this problem became available. Thus, it appeared reasonable that it was the precursor form of the

30 kDa protein that shared many of the characteristics of the labile protein factor described by Garren *(41–44)*.

Proteins that are probably identical to those described by Orme-Johnson have also been characterized in luteinizing hormone (LH) or cyclic adenosine monophosphate (cAMP) analog treated MA-10 mouse Leydig tumor cells by Stocco and colleagues *(65,71–76)*. They also focused on newly synthesized proteins localized to the mitochondria and identified four forms of a 30 kDa protein with isoelectric points ranging from 6.7–6.1 *(64,65,71–76)*. Tryptic peptide analysis indicated that all four proteins were identical, and that phosphorylation was the contributing factor for the more acidic forms of two of the proteins *(64,65,73)*. Precursor proteins of approx 37 and 32 kDa were detected when mitochondrial import was blocked and pulse-chase experiments confirmed the 32–30 kDa precursor to product pathway *(64)*. The hormone-induced synthesis of the 30 kDa proteins paralleled steroid production in both a time and dose-responsive manner and was sensitive to cycloheximide. In support of their role in steroidogenesis, the 30 kDa proteins were found to be maximally expressed in the constitutive steroid-producing rat R2C Leydig tumor cell line where steroidogenesis is independent of trophic hormone activation *(65)*. Further, a tight association between the 30 kDa proteins and steroid production was shown using dimethylsulfoxide (DMSO). Whereas DMSO did not effect general protein synthesis or result in global changes in cAMP-mediated phosphorylation, it was shown to inhibit progesterone production in MA-10 cells, but not in R2C cells *(74)*. The observed difference in these cell lines was the inhibition of expression of the 30 kDa proteins in DMSO-treated MA-10 cells, but not in DMSO-treated R2C cells. Furthermore, the 30 kDa proteins were no longer constitutively expressed, but were synthesized in response to hormone in a clonal population of R2C cells that were selected for reduced basal levels of steroid production and regained responsiveness to hormone stimulation *(75)*. Lastly, expression of the 30 kDa protein was inhibited in MA-10 subclones engineered to overexpress a mutant type 1 regulatory subunit of cAMP-dependent protein kinase A (PKA) or to constitutively overexpress a cAMP-phosphodiesterase *(76)*. These data all suggested that the synthesis of the 30 kDa proteins were dependent upon a functional cAMP-dependent PKA signaling pathway and were intimately linked to the steroidogenic potential of these cells. However, it was clear that these observations were essentially correlative in nature and a direct cause and effect relationship between 30 kDa protein expression and the regulation of steroidogenesis had not been made.

Cloning the Mitochondrial 30 kDa (StAR) Protein

In order to obtain more than correlative evidence for the role of the StAR protein in acutely regulated steroid production, it became necessary to clone its cDNA. First, the 30 kDa protein was purified from stimulated MA-10 cells, and amino acid sequences were obtained for three tryptic peptides. A 400-bp specific product from a MA-10 mouse Leydig tumor cell cDNA library using degenerative oligonucleotides and PCR was then obtained. Using the PCR-generated product to probe a cDNA library, a 1456-bp cDNA was isolated, which contained an open reading frame of 852 nucleotides that encoded a protein of 284 amino acids with a calculated molecular weight of 31.6 kDa *(77)*. Searching several data bases (GenEMBL and SWISS-PROT, GCG Package, University of Wisconsin), no similarities with other nucleotide or protein sequences were found indicating the 30 kDa protein represented a novel protein. In an in vitro transcription/translation system this cDNA was shown to encode the 37 kDa precursor protein, which could be imported and correctly processed and modified to the mature 30 kDa proteins by isolated mitochondria *(77,78)*. Also, the cDNA-encoded and mitochondrially processed protein(s) had identical sizes and mobilities (pIs) as the hormone-induced proteins observed in MA-10 mouse Leydig tumor cells when separated by 2-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) *(77)*.

Transfection Studies with the StAR Protein

The crucial observation that expression of the cDNA-derived protein resulted in increased steroid production in MA-10 cells in the absence of hormone stimulation was made during transient transfection experiments *(77)*. Similarly, when COS-1 cells were cotransfected with P450*scc*, adrenodoxin, and the cDNA for the 37 kDa precursor protein, a sixfold increase in the conversion of cholesterol to pregnenolone was observed *(3,79)*. These results transcended the previous correlative studies and indicated a direct role for the 30 kDa proteins in hormone-regulated steroid production. It was at this time the protein was named the Steroidogenic Acute Regulatory (StAR) protein *(77)*.

Mutations in the StAR Gene Cause Lipoid CAH

Shortly after the cloning of the murine StAR cDNA, the cloning of the human StAR cDNA paved the way for perhaps the most compelling evidence for the importance of StAR in the acute regulation of steroidogenesis. This evidence was obtained from studies on the disease congenital lipoid adrenal hyperplasia (lipoid CAH). Lipoid CAH is a lethal condition that results from an almost complete inability of the newborn infant to synthesize steroids. The lack of functional steroids results in death within days to weeks of birth if not detected and treated with adequate steroid hormone replacement therapy. In this condition, large adrenals containing high levels of cholesterol and cholesterol esters and testicular Leydig cells with an increased amount of lipid accumulation are found. Careful studies by Miller and colleagues demonstrated that mitochondria from adrenals and gonads of affected patients

could not convert cholesterol to pregnenolone *(80–83)*. Therefore, this disease was understandably thought to be the result of an abnormality of P450*scc* enzyme activity, which converts cholesterol to pregnenolone *(81)*. Further studies by Miller's group determined this enzyme was normal in patients who suffered from this disease *(84)*, and it was established that the defect lay upstream of P450*scc* at the point of cholesterol delivery to the enzyme. Additionally, the mRNA and/or protein levels of other factors that either play a role or have been proposed to play a role in the biosynthesis of steroids such as adrenodoxin, adrenodoxin reductase, the steroidogenesis activator polypeptide (SAP), sterol carrier protein 2 (SCP2), HSP-78, the peripheral benzodiazepine receptor (PBR) and its ligand the diazepam binding inhibitor (DBI), have also been shown to be normal in these patients *(84,85)*. Based on the reported characteristics of the StAR protein, it was also a candidate for being involved in lipoid CAH. Its candidacy was strengthened by the observation that StAR mRNA was expressed normally in human adrenals and gonads, but not in placenta *(83)*. Earlier studies had clearly demonstrated that placental steroidogenesis persists in lipoid CAH *(86)*; therefore, the absence of StAR in the placenta was highly consistent with it being a candidate for causing lipoid CAH. To test this possibility, StAR cDNA was prepared from the testicular tissue of several patients with lipoid CAH, and in all of the original patients analyzed, mutations in StAR were identified by sequence analysis *(87)*. These mutations were confirmed in the genomic DNA and two of them were determined to be C to T transitions and result in the premature insertion of stop codons, which in turn truncate the StAR protein by either 28 or 93 amino acids. The expression of the truncated proteins were confirmed by Western analysis following expression of the mutated cDNAs in COS-1 cells *(87)*. Most importantly, expression of the truncated StAR cDNAs in COS-1 cells indicated that the protein produced was completely inactive in its ability to promote steroidogenesis whereas expression of the normal human StAR protein resulted in an eightfold increase in steroid production. A more recent study has analyzed a patient afflicted with a milder form of lipoid CAH, and has also shown that a mutation in the StAR gene was the cause *(88)*. In this patient, cloned genomic DNA was found to have a T to A transversion in intron 4, 11 bp from the splice acceptor site of exon 5 in the StAR gene. This transversion resulted in most of the StAR mRNA in this patient being abnormally spliced and nonfunctional; however, a small percentage was in fact normal StAR mRNA, and thus a milder form of the disease was seen. In addition to the first reports on StAR mutations causing lipoid CAH, many additional examples of mutations in StAR resulting in this disease are being reported *(89–92)*, and perhaps it is not as rare as previously thought. A synopsis of the data on lipoid CAH and mutations in the StAR gene has recently been compiled *(92)*. This report also indicates that many of the mutations in the StAR gene leading to lipoid CAH can be diagnosed using specific restriction endonucleases, a contribution that is certain to have a significant clinical impact. Interestingly, this same article discusses the intriguing possibility that steroidogenic cells possess both StAR-dependent and StAR-independent steroid production, a concept much deserving of further investigation. To date, mutations in the StAR gene are the only known causes of this potentially lethal disease, and have clearly demonstrated the indispensable role of StAR in the production of steroids.

Expression of the StAR Gene

In order to demonstrate StAR's role in hormone-regulated steroid production, Northern and Western analysis demonstrated that StAR mRNA and protein were induced concomitantly via a cAMP-mediated mechanism within a time frame that paralleled the acute production of steroid hormones in MA-10 mouse Leydig tumor cells *(93)*. Furthermore, StAR mRNA and protein expression were shown to be specific to the adrenal, testis, and ovary in the human and mouse, respectively *(79,93)*. The profile of StAR expression during development, as shown by *in situ* hybridization analysis in embryonic mice, also demonstrated a precise spatial and temporal relationship between the presence of StAR transcripts and the capacity to produce steroid hormones *(93)*. In that study, StAR expression during mouse embryogenesis mimicked that previously shown for P450*scc* and the transcription factor Steroidogenic Factor 1 (SF-1) in that StAR was detected in the developing adrenal and testis, but absent in the developing ovary.

To date, many of the studies on the mechanisms and/or treatments that regulate StAR expression are descriptive. Northern analysis has been used to determine the temporal and tissue-specific expression of StAR mRNA and indicate the hormone-induced expression of StAR in MA-10 mouse Leydig tumor cells and human granulosa cells occurs as a result of cAMP-induced changes in StAR transcription and/or mRNA stability *(79,93)*. Juengel et al. *(94)* demonstrated that hypophysectomy resulted in decreased serum progesterone levels and corpus luteum StAR mRNA levels, both of which could be restored to control levels by treatment with the luteotropic hormones LH or GH. On the other hand, prostaglandin F2α ($PGF_{2\alpha}$) and PMA, agents which cause luteal regression, were both shown to decrease StAR transcripts. Pescador et al. *(95)* showed that expression of StAR mRNA and StAR protein were tightly coupled in bovine corpora lutea, both being present at low levels during luteal development, rising during midluteal phase, and disappearing in regressed corpora lutea, a pattern consistent with steroid production. They also demonstrated for the first time that unlike the situation in the human *(79)*, StAR was present in the bovine placenta *(95)*. In yet another study performed in cattle, Soumano and Price found that StAR mRNA expression was tightly coupled to progester-

one production, but not estradiol production in follicular thecal cells *(96)*. In this study they showed that treatment of cows with equine chorionic gonadotrophin (eCG) resulted in an increase in both serum progesterone and estradiol, whereas FSH treatment resulted in only an increase in estradiol. That StAR expression was stimulated by eCG, but not follicle stimulating hormone (FSH) indicates that StAR may be the key component in increased follicular progesterone, and possibly estradiol secretion seen during ovarian stimulation. In yet another example of StAR's role in the physiological maintenance of steroid hormone levels, in the rabbit, in which the luteotropic agent is estradiol, the serum progesterone levels and the corpus luteum content of StAR protein were both shown to be dependent on the presence of estradiol *(97)*. This indicated for the first time that StAR gene expression may also be regulated by a steroid hormone, but as yet no consensus ERE has been located in the 5' promotor region. Also, in the rat ovary Sandhoff and McLean *(98)* demonstrated that both StAR expression and serum progesterone levels increased in parallel in response to trophic hormone stimulation, whereas P450*scc* mRNA levels were unchanged. These same authors later demonstrated that the expression of StAR mRNA in the rat ovary was greatly inhibited by $PGF_{2\alpha}$, the agent believed to be responsible for the regression of the corpus luteum following a nonfertile cycle as mentioned above *(99)*. Balasubramanian et al. *(100)* found that in porcine granulosa cells, progesterone synthesis, StAR mRNA and StAR protein were all tightly coupled and increased in response to a combination of both FSH and insulin-like growth factor-1 (IGF-1), but not to either agent alone. In a clever series of experiments, Selvaraj et al. *(101)*, showed that StAR expression could be stimulated by gonadotrophins in immortalized rat granulosa cells that had been transfected with SV40 DNA containing either LH or FSH receptor genes, as well as by isoproterenol in cells transfected with the β_2-adrenergic receptor gene. Liu et al. *(102)* showed that StAR mRNA was expressed abundantly in both normal human adrenals and in adrenocortical neoplasms. They illustrated that whereas the expression of StAR could be stimulated by ACTH and cAMP analog, the stimulation of expression was low at acute times only reaching a maximum after 24 h. Whereas slower than that seen in other culture systems, the appearance of StAR as well as P450*scc* paralleled that of cortisol production and, as speculated by the authors, may be tied to the time it takes for those cells to differentiate into highly steroidogenic cells in culture. In addition, Nishikawa et al. *(103)* showed a close correlation between ACTH stimulation and StAR protein expression in bovine adrenal fasciculata cells. They also suggested that StAR expression may be regulated by both PKA and PKC signaling pathways in this system. Recently, Kiriakidou et al. *(104)*, demonstrated that StAR messenger RNA is expressed in the most steroidogenic compartments of the human ovary, is expressed in luteinized granulosa cells in response to the LH surge, and its expression is antagonized by activators of protein kinase C. Thus, changes in StAR expression have been shown to be reflective of the physiological changes in steroid secretion in many biological systems that have been studied.

When expressed, different patterns of StAR mRNA have been observed. Three transcripts specific for StAR mRNA have been detected in the mouse [1.6 kb, 2.7 kb, and 3.4 kb] *(77)*, rat [1.2 kb, 1.7 kb, and 3.4 kb] *(105)*, and human [1.6 kb, 4.4 kb, and 7.5 kb] *(79)*, whereas two were detected in the cow [1.8 kb and 3.0 kb] *(95,106)* and pig [1.5 kb and 2.8 kb] *(107)*, and one [2.8 kb] *(94)* in the sheep. It is possible that the difference in length may be attributed in part to a difference in the length of the 3' untranslated regions (B. J. Clark, unpublished observations; also ref. *106*), but the functional significance of the different sized transcripts is not yet known.

Regulatory Elements Involved in StAR Expression

To determine the nature of the cis and trans elements that may be involved in regulating StAR gene expression, approx 1 and 1.3 kb of the 5' flanking regions of the mouse and human gene, respectively, have been isolated and sequenced *(79,93,108,109)*. The murine StAR promoter lacks a canonical TATA box and does not contain a consensus cAMP-responsive element. Thus, like most of the cytochrome P450 steroid hydroylase genes, which also are regulated by cAMP, but lack classical CREs, the regulatory regions of StAR may be unique (rev. in *110*). SF-1 (also known as adrenal 4 binding protein, AD4BP), is an orphan nuclear receptor that has been shown to play key regulatory roles in several different levels of the hypothalamic-pituitary-steroidogenic tissue axis. SF-1 binding sites are present in the promoters of all the steroid hydroxylase genes, and this transcription factor has been shown to transcriptionally regulate their expression in a cAMP-dependent manner *(111,112)*. Being involved in the steroidogenic machinery and because of the temporal relationship observed between SF-1 and StAR during mouse development *(93)*, it was possible that StAR might represent another target gene for SF-1 regulation. Whereas the nucleotide sequence in the coding regions of the StAR gene in the human and mouse share a high degree of similarity, this is not as true for the 5' flanking regions of the genes. However, both the human and mouse StAR promoters contain putative SF-1 binding sites in their 5' promotor regions *(79,108,109)*. A report on the activity of the human promoter has demonstrated the presence of a distal consensus SF-1 binding site and a proximal consensus estrogen receptor binding half site that can confer both basal and cAMP-dependent transcriptional activation of a luciferase reporter gene in Y1 mouse adrenal cells *(109)*. Apparently, both sites are active in regulating human StAR expression, but appear to do so through two different types of interaction.

In a more recent study, it has been demonstrated that the mouse promotor region also contains putative SF-1 binding sites *(108)*. Examination of a 966 bp StAR 5'-flanking region demonstrated that StAR gene expression could be fully regulated by sequences contained within the -254 to -113 region. This finding was in keeping with the belief that SF-1 may be involved in StAR regulation, since a putative SF-1 site was detected at position -135 of the 5' promotor region. It was also determined that induction of StAR transcription could be blocked by actinomycin D, but not by inhibitors of protein synthesis. Site directed mutagenesis of the -135 site revealed that basal promotor activity could be decreased by 50%, but most interestingly, this same mutation had no effect on cAMP-induced expression indicating that the hormonal regulation of StAR expression occurs through an as yet unknown mechanism that may include different promotor elements *(108)*. This latter observation establishes the challenge to determine which elements of the promotor region are required for increased StAR transcription in response to hormone stimulation.

StAR Is Highly Conserved in Different Species

Full length cDNA clones for StAR have been isolated for the mouse *(77)*, rat *(105)*, human *(113–115)*, and bovine *(106)*, and all have greater than 84% homology. The structural gene for StAR has been isolated and characterized for both the mouse and human *(93,113)*. The genes span 6.5 kb in the mouse and 8 kb in the human with the intronic sequences contributing to increased length in the human. Both are organized into seven exons and six introns with exons III-XI being of identical size. A StAR pseudogene was identified by reverse transcriptase polymerase chain reaction (RT-PCR) amplification of RNA from human testis and PCR amplification of human genomic DNA *(113)*. Southern blotting of somatic cell hybrids followed by fluorescent *in situ* hybridization was used to map the human structural gene to chromosome 8p.11.2, and the pseudogene to chromosome 13 *(113)*.

In the species in which the StAR cDNA has been cloned and sequenced and amino acid sequence determined StAR is highly conserved. For example, the mouse and rat StAR amino acid sequences are 96% identical *(105)*, the human and mouse sequences are 87% identical, and 92% similar *(115)*, and the bovine and mouse amino acid sequences 84% homologous *(106)*. In addition, a partial cDNA has been isolated from the sheep that has 80% identity with the corresponding regions of the other species *(94)*. Also, a 280 bp partial cDNA in the pig was shown to be 89%, 91%, and 87% identical to the same regions in the mouse, sheep, and human StAR cDNAs, respectively *(116)*. In general, an 85–90% identity and >90% similarity in StAR has been seen in the various species studied to date. Of the sequences published, the greatest divergence appears to be in the putative mitochondrial signal sequence cleavage site. The mouse sequence contains an amino acid motif that is highly conserved in presequences that undergo a sequential two-step cleavage by the matrix processing protease and the mitochondrial intermediate processing peptide, respectively *(117)*. This putative two-step cleavage site does not appear to be present in the sequences of other species *(105,106,114)*.

Mitochondrial Import and Localization of StAR

As stated, StAR is synthesized in the cytosol and imported and processed by the mitochondria. Interestingly, rat heart mitochondria were used for the import assay with bovine StAR indicating import of StAR is not dependent upon factors specific to mitochondria isolated from steroidogenic tissues *(115)*. This observation is consistent with the results obtained from the functional studies on both murine and human StAR using the COS-1 (monkey kidney) cells in which StAR is imported and processed to its mature forms *(3,77,113)*. Although the mechanism for StAR import and processing remains to be directly determined, the submitochondrial localization of StAR has been determined using protein-A gold labeling of immunoreacted StAR in mouse adrenal zona fasciculata cells. Colloidal gold particles were concentrated within the mitochondria to the intermembrane space and the intermembrane space side of the cristae membrane *(78)*.

StAR Expression and Steroid Synthesis Are Tightly Coupled

In addition to the correlations noted between StAR expression and steroid biosynthesis listed above, several other studies have also clearly demonstrated this tight association. For example, a study in MA-10 cells demonstrated that the organophosphate cholesteryl hydrolase inhibitor, diethylumbelliferyl phosphate, which inhibits steroidogenesis at low concentrations by blocking the transfer of cholesterol into the mitochondria, also inhibited the synthesis of the StAR protein at these same concentrations *(118)*. Also, treatment of mice with endotoxin was shown to result in a very rapid decrease in serum testosterone to 10% of normal levels within 2 h *(119)*. Whereas at this time the steroidogenic enzymes P450*scc*, 3β hydroxysteroid dehydrogenase (3β HSD), and 17α-hydroxylase/C_{17-20} lyase P450 (P450c17) were all shown to be present at normal levels, the StAR protein levels were found to be approx 10% that of animals receiving no endotoxin *(119)*. Choi and Cooke *(120)* found that exclusion of extracellular chloride ion from the medium resulted in the enhancement of steroid synthesis in cultured Leydig cells in response to submaximal, but not maximal doses of trophic hormone. In yet another example of the correlation between steroid synthesis and StAR expression, a potential answer to this observation was recently provided by Ramnath et al. *(121)*. They demonstrated that in MA-10 mouse Leydig tumor

cells, the expression of the StAR protein was enhanced in chloride-free, but not chloride-replete medium in response to submaximally stimulating levels of cAMP analog *(121)*. No effects on the activities or protein levels of either P450*scc* or 3β HSD were seen during this time. Huang et al. *(122)* had earlier reported that corticotropin-releasing factor (CRH) could stimulate cAMP accumulation and steroid biosynthesis in both primary cultures of mouse Leydig cells as well as in MA-10 mouse Leydig tumor cells. In a more recent study, they extended this observation and demonstrated that treatment of MA-10 cells with CRH resulted in a highly significant increase in the accumulation of the StAR protein in a time frame that was tightly correlated with the increase in steroid production *(123)*. Thus, working through the cAMP second messenger pathway, CRH increases StAR synthesis and results in steroid hormone production.

StAR Can Support Steroid Production in Other Systems

In addition to the ability of StAR to increase steroid production when expressed in steroidogenic MA-10 cells, its link to steroidogenesis has been demonstrated in other systems as well. For example, King et al. *(78)* demonstrated that StAR protein obtained from the lysates of StAR transfected COS-1 cells resulted in a time and dose-dependent increase in pregnenolone synthesis when added to mitochondria isolated from MA-10 mouse Leydig tumor cells. This stimulation was shown to be specific for StAR in that pregnenolone synthesis was not affected by addition of another mitochondrial imported protein, adrenodoxin. As mentioned earlier, transfection of the nonsteroidogenic COS-1 cells with StAR cDNA results in a several-fold highly significant increase in steroid production *(3,87,113)*, even though the amount of steroid produced is much lower than that seen in steroidogenic cells. Interestingly, Sugawara et al. *(114)* demonstrated that StAR can stimulate the transfer and metabolism of cholesterol in COS-1 cells cotransfected for expression of the mitochondrial enzyme, cholesterol 27-hydroxylase (CYP27), an enzyme normally found in the liver. Therefore, StAR's function to translocate cholesterol across the mitochondrial membranes is not dependent upon the presence of the cytochrome P450*scc* enzyme.

Phosphorylation and StAR

Given the observation that steroid hormone biosynthesis is increased in response to trophic hormone acting through the cAMP second messenger pathway, questions concerning the role of PKA in this process are natural. Computer analysis of the StAR protein sequence has identified four putative PKA/Cam kinase II phosphorylation sites and one PKC phosphorylation site *(3)*. These consensus sites have been highly conserved in all species whose amino acid sequences have been determined *(3)*. Although it is not known if phosphorylation is a requirement for the mitochondrial import of StAR, the appearance of the mature 30 kDa proteins would not be observed if this process is dependent upon phosphorylation of StAR precursor protein. However, mature, mitochondrial-localized StAR protein has been detected in its unphosphorylated state by 2-D SDS-PAGE electrophoresis in 12–*O*-tertradecanoylphorbol-13-acetate (TPA)-treated MA-10 mouse Leydig tumor cells *(124)*. TPA was shown to induce StAR protein expression without stimulating steroid production. The observation that StAR is not phosphorylated in response to TPA was more recently confirmed in rat adrenal glomerulosa cells, which are also not steroidogenically active in response to TPA *(125)*. However, TPA-treated bovine adrenal fasciculata cells do synthesize steroids, and in this case, the StAR protein was phosphorylated *(125)*. These data would indicate that whereas phosphorylation of StAR itself may not be required for mitochondrial import, phosphorylation of StAR is directly linked to the steroidogenic response to hormone stimulation. Preliminary studies of site-directed mutagenesis of StAR cDNAs at the putative PKA sites have indicated that phosphorylation of one or both of these amino acids (both serines) is important in supporting maximum steroidogenesis in COS-1 cells *(126)*. However, neither the actual phosphorylation site(s) nor their role in supporting steroidogenesis have been unequivocally determined and require further investigation. These data indicate that StAR expression, whereas absolutely required, is not sufficient for steroidogenesis and lend some, albeit indirect, support for phosphorylation as playing a role in StAR's mechanism of action.

Role of StAR in Ca^{2+} Stimulated Steroidogenesis

Elliot et al. *(127)* detected the appearance of several 30 kDa mitochondrial proteins in bovine adrenal glomerulosa cells in response to angiotensin II and K^+ stimulation, agents that increase aldosterone synthesis through the Ca^{2+} second messenger pathway. The molecular weights and isoelectric points of these proteins indicated that they were in all likelihood, StAR. Since that time, StAR was also observed to be induced by angiotensin II (AII) , K^+, TPA, and the calcium channel agonist, BAY K 8644 (BAY K) in the H295R human adrenocortical tumor cell line, thereby confirming that StAR expression may be regulated by the Ca^{2+} signaling pathway as well as the cAMP-dependent second messenger system *(128)*. Furthermore, a dose-dependent inhibition of steroid production has been observed when AII, K^+, or BAY K-stimulated H295R cells are cotreated with a specific inhibitor of Ca^{2+}/CaM-dependent protein kinase II (CaM kinase II) *(129)*. However, the agonist effect on increased StAR expression was not inhibited. The state of StAR phosphorylation was not addressed in these experiments, but four potential CaM kinase II sites are present in StAR's sequence.

In more recent studies, Cherradi et al. *(130,131)* have demonstrated that Ca^{2+} clamping of primary cultures of bovine glomerulosa cells resulted in a cycloheximide sensitive increase in the transfer of cholesterol through mitochondrial contact sites to the inner mitochondrial membrane. It was also demonstrated in the second of these studies *(131)* that Ca^{2+} resulted in a cycloheximide-sensitive increase in StAR protein in the inner mitochondrial membrane. Surprisingly, it was further observed that StAR was also present in the contact site region of the submitochondrial fractions as were the first two enzymes of the steroidogenic pathway, P450*scc* and 3β HSD. These findings confirmed that 3β HSD, as indicated previously *(132,133)*, can be found in the mitochondria as well as in the microsomal compartment. Possible implications of this observation will be discussed later.

Potential Models of StAR Action

As a result of the studies outlined above, it would appear to be clear that StAR has a critical function in the acutely regulated transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Since the mechanism of this transfer is of considerable interest, a model was earlier proposed whereby StAR may act in the transfer of cholesterol to the P450*scc* and has appeared in previous reviews *(1–5,7,8)*. It was proposed that in response to hormone stimulation, the StAR 37 kDa precursor protein was rapidly synthesized in the cystosol and was quickly targeted to the mitochondria via its signal sequence. It was further proposed that as the precursor protein was being imported into the mitochondrial inner compartment and processed, "contact sites" between the inner and outer membranes were formed. During this time, the signal sequence and targeting sequence were sequentially removed by the matrix processing protease and the mitochondrial intermediate processing peptide resulting in the appearance of the 30 kDa mature form of the protein. StAR then remained associated with either the inner mitochondrial membrane or the intermembrane compartment *(78)*. This model further proposed that during the processing of the protein with the accompanying formation of "contact sites" that cholesterol was transferred from the outer to the inner mitochondrial membrane *(18,20,62,64)* and, hence, was available to the P450*scc* for pregnenolone synthesis.

After processing, it was proposed that the membranes separated and no further cholesterol transfer could occur without additional synthesis and processing of StAR precursor proteins. Since the half-life of the precursors of the 30 kDa mitochondrial proteins have been shown to be very short *(62)*, this would explain the observation that steroidogenesis decays very quickly in the absence of new protein synthesis. The fact that StAR was found to be localized to the mitochondria coupled with the observation that the transport of mitochondrial proteins across the membranes occurs at contact sites *(134–139)*, made this a viable model. A model similar to the above was first suggested by Stevens et al. *(140)* who demonstrated that stress-induced ACTH secretion resulted in a dramatic alteration in mitochondrial structure in rat adrenal tissue in which the volume of the matrix compartment increased, but not that of the total mitochondrial volume. This resulted in the outer and inner mitochondrial membranes being brought closer together, a condition that may facilitate the exchange of cholesterol and hence increased steroidogenesis.

However, recent reports by Strauss and colleagues *(141,142)*, have indicated that a revision of this model may be required. It has been shown that *N*-terminal truncations of the StAR protein that remove as many as 62 amino acids have no inhibitory effect on steroid production in COS-1 cells transfected with the cDNAs containing the truncations. Western analysis and immunostaining for StAR protein indicated that the truncated StAR protein was not imported into the mitochondria. Therefore, it appears that import of the StAR protein is not required for cholesterol transfer to the inner mitochondrial membrane. On the other hand, truncation of the C-terminus by 10 amino acids resulted in a decrease in steroid production of 50%, whereas a 28 amino acid truncation resulted in a complete loss of steroid production *(142)*. Thus, it appears that the C-terminal region of the StAR protein is extremely important in cholesterol transfer. This observation could have perhaps been predicted from the observation that all mutations in lipoid CAH have been shown to be in the C-terminal region of the StAR protein. That some of these mutations are single point mutations indicates the powerful role that this portion of the molecule must play in cholesterol transfer *(89–92)*. Perhaps the mechanism of action of StAR in cholesterol transfer requires that it interact with as yet unknown proteins and/or other factors on the outside of the outer mitochondrial membrane and produce alterations that result in cholesterol transfer. This model could still incorporate the formation of "contact sites" between the two membranes and, the idea of specific protein-protein interactions forming hydrophobic channels through which cholesterol can move also remains a possibility. It is further possible that the import of StAR into the inner mitochondrial compartments, which is known to occur with "normal" StAR, may be the "off switch" for steroidogenesis by removing StAR from its position on the outer membrane and cutting off the flow of cholesterol. To further complicate this area, King and Stocco *(143)* have recently shown that both steroid production and StAR synthesis requires ATP hydrolysis and an electrochemical gradient across the inner mitochondrial membrane, a condition shown to be required for mitochondrial preprotein import. Therefore, much remains to be determined concerning the mechanism, whereby StAR can effect cholesterol transfer to the inner mitochondrial membrane. In this regard, the identification of the components with which StAR interacts on the outer mito-

chondrial membrane becomes of critical importance in understanding its mechanism of action.

Though import of StAR does not appear to be an absolute requirement for cholesterol transfer, the finding that import of other mitochondrial proteins does not induce steroidogenesis and that expression of StAR can directly increase steroid output, would suggest a specificity between StAR import and cholesterol transport. In this light, it is especially intriguing that earlier observations have demonstrated that mitochondrial contact sites in bovine adrenocortical cells contain the first two enzymes in the steroidogenic pathway, P450*scc* and 3β HSD *(132)*. Thus, it is tempting to further speculate that the interaction of StAR with the mitochondria may cause the formation of a protein complex consisting of P450*scc* and 3β HSD, the enzymes required for the first two steps in steroidogenesis. In this manner, cholesterol that enters the inner mitochondrial membrane via the action of StAR could quickly be converted to progesterone as speculated in an earlier study *(133)*. The possibility that P450*scc*, 3β HSD, and StAR may all be associated in the same contact sites in steroidogenic cells is supported by the observations of Cherradi et al. *(131)*, which demonstrate by immunoblot analysis that all three proteins, P450*scc*, 3β HSD, and StAR, are found in contact sites isolated from mitochondria of hormone-stimulated bovine glomerulosa cells. It is also possible that the outer mitochondrial membrane protein, the peripheral benzodiazepine receptor, which has been shown to play a key role in steroidogenesis, is also involved in the recognition of StAR by the outer mitochondrial membrane (reviewed in refs. *144–146*). However, at this time these hypotheses are purely speculative and further studies are necessary to confirm the exact relationship between StAR, P450*scc*, 3β HSD and perhaps additional proteins in the mitochondrial membranes.

In summary, the demonstrated characteristics of the StAR protein observed to date make it the most attractive candidate available for the long sought hormone stimulated protein factor responsible for acutely regulating the transfer of cholesterol from the outer to the inner mitochondrial membrane. Additional proteins are unquestionably involved and required in this transfer, but no strong evidence indicates that they are regulatory in nature. Therefore, perhaps the most interesting studies concerning StAR will be to determine the highly specific mechanism whereby StAR is able to effect the transfer of cholesterol to the inner mitochondrial membrane and the P450*scc*. The mechanism of action of StAR in transferring cholesterol to the inner mitochondrial membrane and the roles of other proteins such as SAP, SCP2, PBR and perhaps as yet unidentified proteins remain to be determined and fashion some of the most interesting questions for the future as the picture of the acute regulation of steroidogenesis continues to unfold.

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