COMMENTARY

The role of the StAR protein in steroidogenesis: challenges for the future

D M Stocco

Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, USA
(Requests for offprints should be addressed to D M Stocco; Email: doug.stocco@ttmc.ttuhsu.edu)

Abstract

The steroidogenic acute regulatory or StAR protein has been shown to be instrumental in the acute regulation of steroid hormone biosynthesis through its action in mediating cholesterol transfer to the inner mitochondrial membrane and the cholesterol side chain cleavage enzyme system. Since the time of its cloning in 1994, a number of studies have been performed which underscore the important role that this protein plays in steroidogenesis. While it is now quite apparent that StAR fulfills the criteria for the acute regulator as proposed by early studies, several crucial areas remain poorly understood. This list is

topped by the so far intractable nature of the mechanism of action of StAR in transferring cholesterol to the P450_{scc} enzyme. A second area which should prove to be of great interest is that of further understanding the regulation of the StAR gene which, like many genes, is quite complex. Lastly, with the recent demonstration of StAR being present in the brain, determining if StAR has a role in the synthesis of neurosteroids should prove to be of great importance.

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Introduction

Steroid hormones are synthesized in the adrenals, gonads, placenta and central nervous system. Different steroid hormones having different physiological effects arise from this synthesis, their nature being dependent on the array of enzymes present in the steroidogenic tissue in question. Regardless of tissue origin, however, a common feature of all steroid hormones is that their synthesis utilizes a common precursor, namely, cholesterol. In fact, it is the delivery of cholesterol to the site of its first enzymatic conversion that constitutes the rate-limiting and hormonally regulated step in steroidogenesis (Karaboyas & Koritz 1965, Brownie *et al.* 1972, Simpson *et al.* 1979, Crivello & Jefcoate 1980, Privalle *et al.* 1983, Jefcoate *et al.* 1987). In general terms, cellular cholesterol residing in the outer mitochondrial membrane, lipid droplets or plasma membranes of steroidogenic cells, must be delivered to the inner mitochondrial membrane, the site of the cytochrome P450 side chain cleavage enzyme (P450_{scc}) which converts cholesterol to pregnenolone, the first steroid formed in all steroidogenic tissues. This delivery of cholesterol to the P450_{scc} was determined to be an assisted process, and one that required *de novo* protein synthesis (Ferguson 1962, 1963, Garren *et al.* 1965, 1966).

The identity of the putative acute regulator protein remained a mystery for almost four decades. Although several candidate proteins were proposed, it now appears that the steroidogenic acute regulatory or StAR protein has emerged as the best candidate to fulfill this role. First identified as a 30 kDa phosphoprotein associated with the mitochondria (Krueger & Orme-Johnson 1983, Pon *et al.* 1986, Pon & Orme-Johnson 1988, Stocco & Kilgore 1988, Epstein & Orme-Johnson 1991, Stocco & Sodeman 1991), StAR was purified, cloned, sequenced and expressed in 1994 (Clark *et al.* 1994). Importantly, its expression in MA-10 or COS-1 cells resulted in significant increases in steroid hormone synthesis in the absence of hormone stimulation, thus indicating that StAR could mediate cholesterol transport to the P450_{scc} enzyme (Clark *et al.* 1994, Sugawara *et al.* 1995, Lin *et al.* 1995, Stocco & Clark 1996). Perhaps the most compelling evidence for the indispensable role for StAR in mediating cholesterol transfer and hence regulating steroid hormone biosynthesis was the finding that mutations in the StAR gene result in the potentially lethal condition known as congenital lipoid adrenal hyperplasia (Lin *et al.* 1995). This disease is characterized by an almost complete blockage of steroid hormone synthesis and significantly, this blockage was found to be at the locus of cholesterol delivery to the P450_{scc} enzyme.

More recently, StAR knockout mice have been generated and their phenotype is essentially identical to that seen in the human condition, further demonstrating the role for StAR in steroidogenesis (Caron *et al.* 1997b).

In the past five years, many studies have demonstrated the role of StAR in developmentally and hormonally regulated steroid biosynthesis. A number of previous review articles are available on this subject and more thoroughly summarize studies performed to date (Stocco & Clark 1996, 1997, Stocco 1997a, b, 1998a, 1998b, 1999, Clark & Stocco 1997, Miller 1997, 1998, Cherradi *et al.* 1998). The subject of this short review will be to point out what are, in the opinion of this author, several of the most challenging questions that have emerged concerning the role of StAR in steroidogenic tissues. This list is certainly not meant to imply that other areas are not of interest or importance, but is merely intended to delineate several problem areas that should be of interest to a variety of scientists.

How does StAR transfer cholesterol?

Clearly StAR has an indispensable function in the acutely regulated synthesis of steroids. How does it work? A model was proposed indicating that StAR was synthesized in the cytosol in response to trophic hormone stimulation, and during import into the mitochondrial inner compartment, contact sites between the inner and outer membranes were formed which served as the conduit for outer mitochondrial membrane cholesterol to transfer to the inner membrane (Epstein & Orme-Johnson 1991, Stocco & Sodeman 1991, Jefcoate *et al.* 1992, Stocco & Clark 1996). It was soon clear that this model was incorrect. N-terminal truncations of the StAR protein that removed as many as 62 amino acids and completely inhibited import had no effect on cholesterol transfer or steroid production when transfected into MA-10 or COS-1 cells (Arakane *et al.* 1996, Wang *et al.* 1998). Similar observations were made in a completely *in vitro* system by Arakane *et al.* (1998) who demonstrated that bacterially produced StAR protein lacking the first 62 N-terminal amino acids was able to support full steroidogenesis in isolated mitochondria but was not imported. Conversely, when mitochondria were incubated with StAR protein whose C-terminus was truncated by 28 amino acids, a complete loss of steroid production resulted (Arakane *et al.* 1996, Wang *et al.* 1998). These data all indicate that the C-terminal region of the StAR protein functions in cholesterol transfer.

A further indication of the importance of the C-terminal region of the StAR protein in cholesterol transfer was shown by Watari *et al.* (1997), in describing the steroidogenic properties of a protein, MLN64, which has homology to the C-terminal region of StAR. Expression of MLN64 in COS-1 cells resulted in a twofold increase in steroid production. The relationship between StAR and MLN64 as well as the role of MLN64 in the cell

remain to be determined and hopefully useful information concerning sterol movement in the cell will be obtained.

More recent studies have demonstrated that StAR can act as a sterol transfer protein to enhance sterol desorption from one membrane to another (Kallen *et al.* 1998a). In this model, StAR is directed to the mitochondria via its N-terminus and then, via C-terminal sequences it produces alterations in the outer mitochondrial membrane that results in the transfer of cholesterol from the outer to the inner membrane. This transfer of cholesterol was specific in that identical experiments employing phosphatidylcholine failed to show transfer of this phospholipid. This is pertinent to the situation found in steroidogenic mitochondria in which the desorption of cholesterol from the sterol-rich outer membrane to the sterol-poor inner membrane (Martinez & Strauss 1997) would serve to enhance pregnenolone synthesis by the P450_{scc} enzyme (Kallen *et al.* 1998a).

While the mechanism of action of the StAR protein is still unknown, it seems clear that cholesterol transfer requires that it interact, at least transiently, with components such as proteins, lipids and/or other factors on the outside of the outer mitochondrial membrane and produce alterations which result in cholesterol transfer. The identification of such factors has so far proven to be quite elusive (Kallen *et al.* 1998b), but it appears that StAR promotes cholesterol transfer as a result of its direct interaction with the outer surface of the mitochondria and not through an intermediary (Kallen *et al.* 1998a). This was demonstrated through the use of recombinant StAR protein which, when added directly to purified mitochondria, was shown to increase pregnenolone production. To date, studies designed to identify StAR-interacting proteins have utilized the yeast two-hybrid assay system with StAR as bait, co-immunoprecipitation of StAR expressing COS cell lysates and binding assays using radiolabeled StAR protein incubated with isolated mitochondria (Kallen *et al.* 1998b). None of these approaches has yet identified a legitimate StAR binding partner. Perhaps, as recently speculated (Arakane *et al.* 1998), StAR can stimulate cholesterol transfer, either as a result of a few very high affinity stable interactions with the outer mitochondrial membrane which are difficult to detect because of their low number, or as a result of transient interactions which would also be difficult to detect because of their fleeting nature. To determine how the StAR protein might interact with the outer mitochondrial membrane, Miller and colleagues subjected StAR to limited proteolysis at different pH values and found that the molecule behaves very differently as the pH decreases (Bose *et al.* 1999). They showed that StAR can form a molten globule in the pH 3.5–4.0 range. i.e. if the pH microenvironment surrounding the mitochondria is acidic, the StAR molecule may undergo a conformational shift, forming an extended structure and increasing the flexibility of the linker region located between the

Figure 1 Putative mechanism of StAR action: The model in this figure represents what is currently known about the mechanism of action of StAR. (1) Upon stimulation of the steroidogenic cell with trophic hormone, the 37 kDa StAR protein precursor is rapidly synthesized in the cytoplasm. This precursor is accompanied by chaperone proteins which prohibit folding of the precursor, a condition which would make import of the protein into the mitochondrion impossible. It is also noteworthy that a section of the protein encompassing amino acids 63–188 has been shown to be protease resistant, a feature which may be important in cholesterol transfer. (2) The 37 kDa protein then becomes associated with the mitochondria and the import and processing of the precursor protein begins. At some point, either prior to the start of import, or simultaneously with it, sequences in the C-terminal portion of the StAR protein interact with as yet unidentified components of the outer mitochondrial membrane and result in cholesterol transfer to the inner mitochondrial membrane. The protease resistant portion of the StAR protein results in the formation of a molten globule which may be instrumental in cholesterol transfer. (3) Cholesterol is quickly converted to pregnenolone and in some steroidogenic tissues, to progesterone. (4) Cleavage of the precursor protein by the matrix processing protease results in the formation of the mature 30 kDa form of the StAR protein. (5) StAR is refolded. (6) It becomes associated with the inner mitochondrial membrane and also with the intermembrane space. Once imported StAR is no longer in contact with the outer membrane and further transfer of cholesterol is stopped. Thus, it is the continuous synthesis and processing of precursor StAR to mature StAR, with the continual interaction between the C-terminal part of StAR and the outer mitochondrial membrane which is responsible for cholesterol transfer. Also, it is unknown as to whether or not the 30 kDa form of the protein has any functional role when in the mitochondria. The nature of the components with which StAR interacts on the outer membrane are unknown, and perhaps the peripheral benzodiazepine receptor (PBR) is involved at this site. Also, the mitochondrial contact sites and inner membranes are enriched with the first two enzymes in the steroidogenic pathway, namely P450scc and 3β hydroxysteroid dehydrogenase, raising the interesting possibility that cholesterol entering the mitochondria may leave as progesterone and not pregnenolone in at least some steroidogenic cell types.

N-terminus and the biologically active C-terminus while acting on the outer mitochondrial membrane. As the transition to a molten globule occurs, this structural change may lower the energy required to open the StAR structure further, possibly exposing a cholesterol channel or, it may prolong the interval in which StAR can reside on the outer membrane thus allowing increased transfer of cholesterol during this period.

Very little is known about the manner in which StAR effects cholesterol transfer to the inner mitochondrial membrane. Identification of the components interacting

with StAR on the outer mitochondrial membrane and the nature of this interaction become of critical importance in understanding its mechanism of action. The identification of these putative binding partners has remained elusive and their characterization and the description of their role in cholesterol transfer can be considered as perhaps the most interesting and important undertaking in this field. A highly simplified illustration of what is currently known concerning the mechanism of action of StAR in transferring cholesterol from the outer to the inner mitochondrial membrane is shown in Fig. 1.

Regulation of the StAR gene

The trophic hormone-induced increase in steroid production in steroidogenic cells is accompanied by rapid increases in StAR mRNA levels (Clark *et al.* 1995, Sugawara *et al.* 1995, Caron *et al.* 1997a). Since this is generally considered to be a cAMP-mediated event, attention focused on the role of cAMP in the regulation of the StAR gene (Caron *et al.* 1997a, Sugawara *et al.* 1995, 1996, 1997, Rust *et al.* 1998, Sandhoff *et al.* 1998, LaVoie *et al.* 1999). Like several other cAMP-regulated genes involved in steroid biosynthesis, the promoter region of the StAR gene lacks an easily recognizable cAMP response element. However, studies have shown that the cAMP responsive site is retained within a 245 nucleotide region relative to the transcription start site (Caron *et al.* 1997a). Therefore, this region has been the focus of studies to identify promoter elements and their cognate-binding proteins that could mediate the cAMP response.

A transcription factor considered to be potentially important in the regulation of the StAR gene was steroidogenic factor 1 (SF-1). SF-1 was first identified in adrenal cortical cells, and has been shown to be instrumental in regulating the cytochrome P450 steroid hydroxylase genes (Ikeda *et al.* 1993). Of special interest to studies on StAR function was the finding that SF-1 knockout mice do not express StAR mRNA, indicating that SF-1 is required for proper StAR gene expression (Caron *et al.* 1997a). Several SF-1 consensus binding sites have been found in the StAR promoter. In rats, five SF-1 sites have been reported (Sandhoff *et al.* 1998), and one additional site has been identified in humans and mice (Caron *et al.* 1997a, Sugawara *et al.* 1997). Two of these sites, which are located at positions -97 and -42, are highly conserved in several species whose promoter regions have been sequenced to date. Most importantly, SF-1 has been demonstrated to transactivate the StAR promoter in transient transfection assays in numerous cell types (Caron *et al.* 1997a, Sugawara *et al.* 1996, 1997, Sandhoff *et al.* 1998). SF-1 may also play some role in the developmental regulation of the StAR gene as StAR mRNA is not detected in the urogenital ridge of SF-1 null mice (Caron *et al.* 1997b).

While SF-1 was shown to participate in StAR expression, it was apparent that additional elements were involved in StAR's tissue and temporal specific expression, and the search for additional transacting factors and/or co-regulators involved in the regulation of the StAR gene has begun. The CCAAT/enhancer binding proteins (C/EBPs), are a family of basic region/leucine zipper transcription factors implicated as regulators of differentiation and function of multiple cell types (Johnson & Williams 1994). Previous studies have demonstrated that family members C/EBP α and C/EBP β are expressed in Leydig cells and ovarian granulosa cells (Nalbant *et al.* 1998, Sirois & Richards 1993). We recently identified two

C/EBP binding sites in the StAR promoter of MA-10 Leydig cells in our laboratory (Reinhart *et al.* 1999). We also determined that the StAR promoter is transactivated by C/EBP β and that SF-1 transactivation of the StAR promoter is dependent on the presence of functional C/EBP binding sites, suggesting that SF-1 and C/EBP β form a complex on this promoter. Silverman *et al.* (1999) recently conducted functional assays of the StAR promoter using follicle-stimulating hormone (FSH) induced naive primary granulosa cells from prepubertal rat ovaries. This approach led to the identification of a non-consensus binding sequence for C/EBP β (-81/-72), located just ten nucleotides upstream from a consensus motif for GATA-4 binding (-61/-66). Site-directed mutagenesis of both binding sites essentially eliminated basal and hormone driven StAR expression, reinforcing the notion that both of these elements are required for FSH-induced acute transactivation of the StAR promoter in these cells. Western blot analyses showed that GATA-4 is constitutively expressed in granulosa cells whereas three isoforms of C/EBP β were rapidly induced by FSH. Therefore, it was suggested that while GATA-4 may play a permissive role, C/EBP β could meet the criteria of a transcription factor which could regulate acute StAR transcription in granulosa cells. In another recent study, it has been demonstrated that the sterol regulatory element binding protein (SREBP) may also be involved in the regulation of the StAR gene as it was reported that SREBP-1a was capable of transactivating the StAR promoter (Christenson *et al.* 1998). While no consensus binding sites for SREBP-1a have yet been found in the StAR promoter, this may prove to be an example of the convergence of the regulation of cholesterol metabolism and steroidogenesis. It should be noted that while the transcription factors SF-1, C/EBP β , GATA-4 and SREBP-1a have all been shown to be active in StAR gene expression, the absolute requirements for acute regulation have not yet been fully determined.

In summary, knowledge regarding the regulation of the StAR gene is expanding rapidly and it has become quite apparent that, like many other genes, a host of transcription factors and/or co-regulators are involved in its expression. Since it is becoming abundantly clear that regulation of the StAR gene is a complex process, the biggest challenge in this area will be to characterize those elements that are involved in its acute regulation and those that serve to provide tissue-specific and temporal-specific expression of the gene. Figure 2 depicts a comparison of the promoter region of five different species in which this region has been sequenced. As is readily seen, sequence homology has been highly conserved in several areas of the StAR promoter.

StAR in the brain

Like the highly active steroidogenic tissues, the adrenal gland and the gonads, the central and peripheral nervous

Figure 2 StAR promoter comparisons: Comparison of the sequences of the 5' flanking regions of the mouse (M), rat (R), ovine (O), porcine (P), and human (H) StAR genes were aligned using the ClustalW sequence alignment program and identical bases in all five sequences are indicated with asterisks. Binding sites for SF-1 are shaded and labeled. Two putative C/EBP binding sites were identified based on their similarity to the consensus C/EBP binding site and are shaded and labeled. Other highly conserved sequences discussed in the text are boxed and labeled A and B.

systems also synthesize steroids, which are collectively referred to as neurosteroids (Baulieu 1997). The neurosteroids exhibit a number of physiological functions which include both stimulation and inhibition of GABAergic responses (Majewska *et al.* 1986), modulation of the response of Purkinje cells to excitatory amino acids (Smith 1991) and enhancement of memory function (Flood *et al.* 1992, Mathis *et al.* 1994). The nature of neurosteroid biosynthesis and certainly the mechanisms involved in its regulation are not, however, well understood. Given the purported role of StAR in steroidogenesis, it was of great interest to determine if it was present in central nervous tissue. The first reports detailing the localization of StAR in human and mouse tissues failed

to detect its presence in brain (Clark *et al.* 1995, Sugawara *et al.* 1995). Thus it remained unclear if StAR performed any role in neurosteroid synthesis. It could perhaps be predicted that if StAR were found in the brain, it would be present at much lower levels, given the highly reduced nature of neurosteroid synthesis when compared with adrenals and gonads. Using the sensitive method of RT-PCR, Furukawa *et al.* (1998) have demonstrated that StAR transcripts are indeed present in rat brain, an observation which was confirmed with *in situ* hybridization techniques. StAR transcripts were present in the cerebral cortex, hippocampus, dentate gyrus, olfactory bulb, cerebral granular layer and Purkinje cells. These investigators estimated that the amount of StAR transcripts

present in the brain was two to three orders of magnitude less than that found in the adrenal gland, hence the difficulty in their detection by less sensitive methods. These investigators were also able to demonstrate that StAR transcripts were co-expressed with cytochrome P450_{sc} and 3 β -HSD in the hippocampus, dentate gyrus, cerebral granular layer and Purkinje cells. These results indicate that neurosteroids are synthesized in highly specific regions in the brain and that the StAR protein may be involved in this synthesis. A great deal more work must be performed to make definitive statements on the characteristics of neurosteroid synthesis and the potential role of StAR in this synthesis, but the demonstration that StAR is co-localized with steroidogenic pathway enzymes in the brain should make this an exciting area of study in the future. For example, it will be possible to determine if the StAR knockout mouse suffers from any central nervous system deficiencies. Also, the particularly provocative question of whether or not StAR, and perhaps neurosteroid synthesis, is regulated in the brain can now be addressed.

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