Pergamon

0960-0760(95)00212-X

J. Steroid Biochem. Molec. Biol. Vol. 55, No. 5/6, pp. 607–616, 1995

0960-0760/95 $9.50 + 0.00

Mitochondrial Specificity of the Early Steps in Steroidogenesis

Walter L. Miller

Department of Pediatrics and the Metabolic Research Unit, University of California, San Francisco, CA 94143-0978, U.S.A.

Studies in human beings, animals, and cell systems show that the rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone. In the adrenals and gonads, this step is subject to both acute and chronic regulation. Chronic regulation is primarily, but not exclusively at the level of gene transcription, leading to the production of more steroidogenic machinery and thus increasing the cellular capacity for steroidogenesis. Chronic regulation can be inhibited by inhibiting protein synthesis with cycloheximide, but this response varies among various cell types and species. Although the *P*450scc enzyme system that converts cholesterol to pregnenolone is inherently very slow, the principal site of acute regulation is at the delivery of free cholesterol to mitochondria, rather than at the delivery of reducing equivalents to *P*450scc. Even when the V_{max} of the *P*450scc system is increased 6-fold by genetic engineering, delivery of cholesterol to the enzyme remains rate-limiting. Targeting of a genetically engineered fusion of the *P*450scc system to either mitochondria or to the endoplasmic reticulum of non-steroidogenic cells demonstrates that the mitochondrial environment is absolutely required for the conversion of cholesterol to pregnenolone, and that this absolute requirement is not based on either the nature of the available electron donors for *P*450scc or the availability of substrate. Various factors have been proposed as the essential mediator for the transport of cholesterol into mitochondria to initiate steroidogenesis. A recently identified protein termed Steroidogenic Acute Regulatory protein (StAR) has the necessary properties of enhancing steroidogenesis, rapid cAMP inducibility and rapid cycloheximide sensitivity that characterize the long-sought acute regulator of steroidogenesis. StAR is expressed in steroidogenic tissues exhibiting an acute response but not in steroidogenic tissues (placenta, brain) that do not exhibit this response. Mutations in StAR are now shown to cause Congential Lipoid Adrenal Hyperplasia, the last unsolved form of CAH. The actions of StAR can be circumvented by the use of hydroxycholesterols that can freely diffuse into mitochondria, proving that StAR functions as an acute regulator of cholesterol access to mitochondria.

J. Steroid Biochem. Molec. Biol., Vol. 55, No. 5/6, pp. 607–616, 1995

INTRODUCTION

Physiologic studies in intact animals and human beings have long recognized two forms of regulation of the adrenal secretion of glucocorticoids: the acute and chronic responses. The acute response is very rapid. When ACTH is administered intravenously to normal human beings, plasmid cortisol concentrations begin to rise within 15 min and are maximal by 60 min. This response entails the activation of preexisting adrenal steroidogenic machinery, and thus can be used as a measure of adrenal steroidogenic reserve (for a full discussion see [1]). By contrast, the chronic response is slow. Administration of ACTH for days and weeks will result in an increase in adrenocortical mass, cell number (hyperplasia), cell size (hypertrophy) and of the absolute amount of the steroidogenic enzymes present per cell. Thus the chronic response entails adrenal growth and the synthesis of more steroidogenic machinery per cell. ACTH-induced adrenal cell growth and induction of steroidogenesis are separate events. Human fetal adrenal cell growth is mediated by insulin-like growth factor II [2] in conjunction with basic fibroblast growth factor [3] and possibly epidermal growth factor [4], but ACTH-induced adrenal cell growth in adult rats does not appear to be dependent on IGF-I or IGF-II [5]. However, as the chronic

Proceedings of the Workshop on the Molecular and Cell Biology of Hydroxysteroid Dehydrogenases, Hannover, Germany, 19–22 April 1995.

response entails an increase in the synthesis and accumulation of steroidogenic enzymes per cell, the cell growth component does not need to be considered in examining the chronic regulation of the steroidogenic enzymes.

When the adrenal is stimulated, either chronically or acutely, the rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone [6–11]. Conversion of cholesterol to pregnenolone involves three distinct steps: 20α-hydroxylation, 22-hydroxylation, and cleavage of the bond between carbon atoms 20 and 22 on the cholesterol side-chain, yielding pregnenolone and isocaproaldehyde. All three of these reactions occur in the single substrate-binding pocket of *P*450scc, a Type I (mitochondrial) cytochrome *P*450scc enzyme [12]. Like all Type I *P*450 enzymes, *P*450scc must receive electrons from NADPH through the intermediacy of two electron transfer proteins. Ferredoxin reductase, also termed adrenodoxin reductase, is a flavoprotein that accepts electrons from NADPH and then transfers them to ferredoxin, also termed adrenodoxin, an iron/sulfur protein that then interacts with the *P*450scc. It is the *P*450scc that catalyzes the conversion of cholesterol to pregnenolone, and hence this enzyme (or its mRNA) can only be found in steroidogenic tissues [13, 14]. By contrast ferredoxin and ferredoxin reductase are generic electron transfer factors used by all type I *P*450 enzymes, and hence these proteins and their mRNAs are found in every tissue examined [15–17]. Thus the chronic induction of steroidogenesis is mediated by the synthesis and accumulation of these three proteins.

THE CHRONIC RESPONSE

Numerous experiments, both in whole animals and in cell culture systems, have shown that inhibition of protein synthesis with cycloheximide can inhibit both basal and hormonally-stimulated steroidogenesis. Experiments with bovine adrenal cells showed that the mRNAs for various steroidogenic enzymes could accumulate within 4 h of stimulation with ACTH or cAMP, that this stimulation increased gene transcription, and that this increased gene transcription could be blocked by cycloheximide [18]. These results suggested that the cycloheximide-sensitive "labile factor" required for ACTH-induced steroidogenesis was a transcription factor termed steroid-hormone inducing protein [10]. However, other results were inconsistent with this hypothesis. The gonadotropin or cAMP-induced accumulation of mRNAs for *P*450scc and adrenodoxin in human granulosa was not inhibited by cycloheximide [19], while in human placental cytotrophoblasts cycloheximide did inhibit cAMP-induction of *P*450scc but potentiated the induction of adrenodoxin [16, 20]. Similarly cycloheximide potentiated the cAMP-induced accumulation of adrenodoxin mRNA but has no effect on *P*450scc gene transcription in mouse Leydig MA-10 cells [21, 22]. Thus the effects of cycloheximide on the chronic steroidogenic response are variable, depending on the species, tissue, and gene examined. It now appears clear from intensive investigation of the transcription of the genes for the steroidogenic enzymes that there is at least one factor, steroidogenic factor I (SF-1) that is required for the transcription of all these genes [23–26], but that such transcription factors are not the basis for the cycloheximide sensitivity of the steroidogenic response to tropic factors.

In most systems, the chronic induction of the synthesis of a specific protein is mediated at the level of increased gene transcription. This appears to be the case for adrenodoxin, which has only been studied in human placental cells and mouse adrenal cells [27, 28] and is clearly the case for *P*450scc, where the human, bovine, and rat genes have been studied in many different tissues (for reviews see [29, 30]). By contrast adrenodoxin reductase appears to represent a special case. The accumulation of adrenodoxin reductase protein is hormonally inducible in human placental cells and mouse adrenal cells [31] but the transcription of the adrenodoxin reductase gene appears to be constitutively turned on at high levels, and that the action of cAMP is to reduce the abundance of the corresponding mRNA post-transcriptionally, by unknown means [17].

Studies of *P*450scc gene transcription are especially illuminating. The gene is clearly inducible by cAMP in many species and tissues [18, 22, 27, 28, 32–40]. However, the human *P*450scc gene is induced by cAMP in two completely different ways in different tissues. In the adrenals and gonads, induction by cAMP is mediated by a typical cAMP-response element (CRE) located 1620–1676 bases from the transcriptional start site [27, 28, 34, 35, 39], whereas placental cAMP responsiveness is mediated by sequences between 89 and 108 bases from the cap site, which bear no resemblance to those between −1620 and −1676 [28, 36, 40]. Similarly, the adrenal and gonadal expression of *P*450scc requires sequences between −110 and −127 [30, 34, 39] but these sequences do not function in placental cells, which instead use sequences between −142 and −153 to achieve placental-specific expression [27, 28, 36, 40]. Thus *P*450scc transcription in the adrenals and gonads, the steroidogenic tissues that derive from the embryologic gonadal ridge [41], differs fundamentally from *P*450scc transcription in the placenta. Similarly, while cycloheximide diminishes *P*450scc gene transcription and mRNA accumulation in human placental [37] and bovine adrenal cells [18], cycloheximide has no effect on adrenodoxin gene transcription and induces adrenodoxin mRNA accumulation in human placental cells [37] while it reduces adrenodoxin gene transcription in bovine adrenal cells [18]. Thus fundamental differences are found in the biochemical basis of the chronic response in placental versus adrenal/gonadal cells. As we shall

see below a similar fundamental difference is seen in the acute response in these two classes of cells as well.

EFFICIENCY OF P450*scc*

The conversion of cholesterol to pregnenolone in the mitochondria of steroidogenic cells is the first and rate limiting step in steriodogenesis. Because this step is both rate-limiting [42] and hormonally regulated [2, 13, 43–47] it has been the subject of intense study. The conversion of cholesterol to pregnenolone entails three distinct steps: 20α-hydroxylation, 22-hydroxylation and scission of the C20–22 carbon bond, all in the single substrate-binding pocket of *P*450scc. This is an extremely slow reaction, requiring about 1 s for a molecule of *P*450scc to convert a single molecule of cholesterol to pregnenolone [48], so that the reaction intermediates, 20α-hydroxycholesterol, 22-hydroxycholesterol and 20,22 dihydroxycholesterol, can be isolated in appreciable quantities. Two possible explanations can be offered for the unusually slow rate of this reaction, either the availability of the cholesterol substrate is limiting or the availability of reducing equivalents from NADPH is limiting. Early studies focussed on the availability of cholesterol and its acute stimulation by ACTH and cAMP, i.e. the acute response.

Unlike the chronic response, where cycloheximide induces different effects on various genes in different species and cell types, cycloheximide appears to abolish the acute adrenal and gonadal response in all systems examined [44, 49–52]. This acute inhibition does not appear to affect the *P*450scc enzyme system directly, but rather blocks the movement of cholesterol from the outer mitochondrial membrane, which is in contact with the cytosol, to the inner mitochondrial membrane, which harbors the *P*450scc system [53–55]. Thus many studies indicate that the rate-limiting step in steroidogenesis is the availability of cholesterol to the *P*450scc enzyme, rather than the behaviour of the enzyme itself.

However, other data suggest that the availability of reducing equivalents can be limiting. For example, when non-steroidogenic COS-1 monkey kidney cells are transfected with vectors expressing bovine or human *P*450scc, the endogenously available adrenodoxin reductase is adequate to support maximal *P*450scc activity, but the endogenously available adrenodoxin is not. Co-transfection with vectors expressing adrenodoxin will amplify *P*450scc activity substantially [56, 57]. Double transfection of vectors expressing *P*450scc and adrenodoxin reductase yields no more cholesterol side-chain cleavage activity than transfection with *P*450scc alone, and triple transfection with all three components yields no more activity than double transfection with *P*450scc and adrenodoxin [57]. Thus the abundance of adrenodoxin can substantially regulate the activity of a constant amount of *P*450scc. *P*450scc, adrenodoxin and adrenodoxin reductase are all associated with the matrix surface of the inner mitochondrial membrane, and can freely move on this surface, as they are not found in a fixed stoichiometric ratio and are never found as a ternary (3-component) complex [55, 58, 59]. Thus both the abundance and diffusability of adrenodoxin within the mitochondrion appear to be able to regulate *P*450scc activity.

In addition to the failure to observe ternary complexes by electron microscopy [55] theoretical considerations indicate that a ternary complex could not function, as the same surface of the adrenodoxin molecule appears to interact with both *P*450scc and adrenodoxin [58, 59]. To determine if such a ternary complex could function and to bring the adrenodoxin moiety into intimate contact with the *P*450scc, we built a series of vectors that express monomolecular fusion proteins consisting of human *P*450scc, adrenodoxin, and adrenodoxin reductase in a variety of organizations [57, 60] (Fig. 1). In all cases, the *P*450scc moiety was positioned at the amino terminus because this is the arrangement in the naturally occurring Type II *P*450

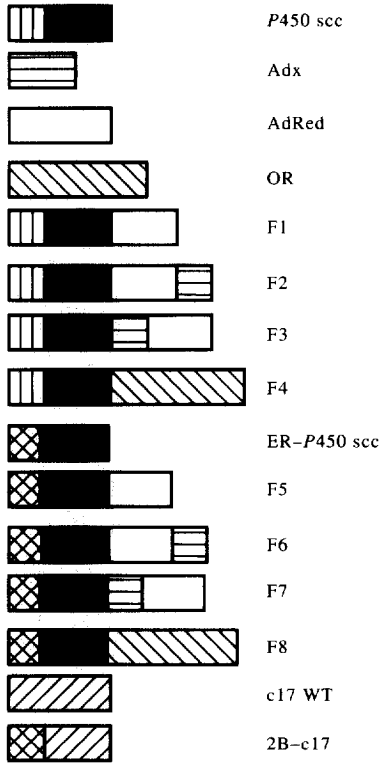

Fig. 1. Constructions used in this study. Leader sequences at the amino-terminus (5′ end, left) are the 39 amino acid mitochondrial leader sequence of human *P*450scc (▥), or the 23 amino acid microsomal (endoplasmic reticulum) leader sequence of rat *P*450IIB1 (▩). Coding regions follow the leader sequences: ■, *P*450scc; ▤, adrenodoxin (Adx); □, adrenodoxin reductase (AdRed); ▧, *P*450 oxidoreductase (OR). The *P*450c17 constructions are shown at the bottom. c17WT expresses the wild-type human *P*450c17 protein (▨), and 2B-c17 has the same *P*450IIB1 microsomal leader sequence used in ER–*P*450scc and F5–8. Also shown in this diagram are the constructions expressing wild-type human adrenodoxin and adrenodoxin reductase, which use their own endogenous mitochondrial leader sequences, and the construction expressing human *P*450 oxidoreductase, which uses its own endogenous microsomal leader sequence. For further details see [60].

fusion protein *P*450BM3 of *Bacillus megaterium* [61–63] and appears to be the only effective location in artificially created fusions of Type II *P*450 enzymes [64]. Thus the mitochondrial leader sequence of *P*450scc was retained while the leader sequences of adrenodoxin and adrenodoxin reductase and the 3′ untranslated regions of all three mRNAs were deleted [57]. These constructions were cloned into an expression vector, transfected into non-steroidogenic COS-1 cells and incubated with 22R-cholesterol, a polar, freely diffusible cholesterol analogue which does not require a mitochondrial cholesterol transport system. Initial studies transfecting COS-1 cells with the constructions F1, F2 and F3 (Fig. 1), or triply transfecting these cells with equimolar amounts of vectors expressing *P*450scc, adrenodoxin and adrenodoxin reductase individually showed that the F2 construct generated substantially more pregnenolone than any of the other systems [Fig. 2(a)]. Kinetic analysis showed that the F2 fusion enzyme had a V_{max} of 9.1 ng pregnenolone per ml of culture medium per 24 h, compared to 1.7 for the triply transfected cells [Fig. 2(b)]. Thus the F2 fusion construction is a superior cholesterol side-chain cleavage enzyme that permits one to study the *P*450scc system in a covalently fixed 1:1:1 stoichiometric ratio of the three components of the system.

It has been reported that cholesterol side-chain cleavage activity can be found in microsomes and cytosol, as well as in mitochondria (for review and references see [11]). Because all cellular fractionation and protein purification experiments are subject to contamination, a definitive test of the location of *P*450scc cannot be done by disrupting cells. Thus we have investigated the requirements for the mitochondrial environment for *P*450scc function by placing appropriate leader sequences upstream from the protein-coding region of vectors that then target the expressed protein to mitochondria or to the endoplasmic reticulum in intact cells [60]. By adapting this approach to the F2 fusion, we can direct the *P*450scc enzyme and its attendant electron transfer proteins to the endoplasmic reticulum, and maintain the three components in a fixed stoichiometric ratio and in a functional spatial relationship to one another. When mitochondrial *P*450c27 is targeted to the endoplasmic reticulum of yeast co-transfected with vectors that express adrenodoxin and adrenodoxin reductase in soluble, cytoplasmic forms, the *P*450c27 is active [65] suggesting that *P*450scc might also be active when targeted to the endoplasmic reticulum. By using 22-hydroxycholesterol as a substrate, rather than cholesterol, we can ensure that the substrate is freely accessible to all cellular compartments and is not subject to an active mitochondrial transfer system, as is the case with cholesterol [44, 66]. Finally, we could test whether the nature of the electron donor was crucial by re-engineering the vectors to substitute the microsomal flavoprotein *P*450 oxidoreductase that is used by

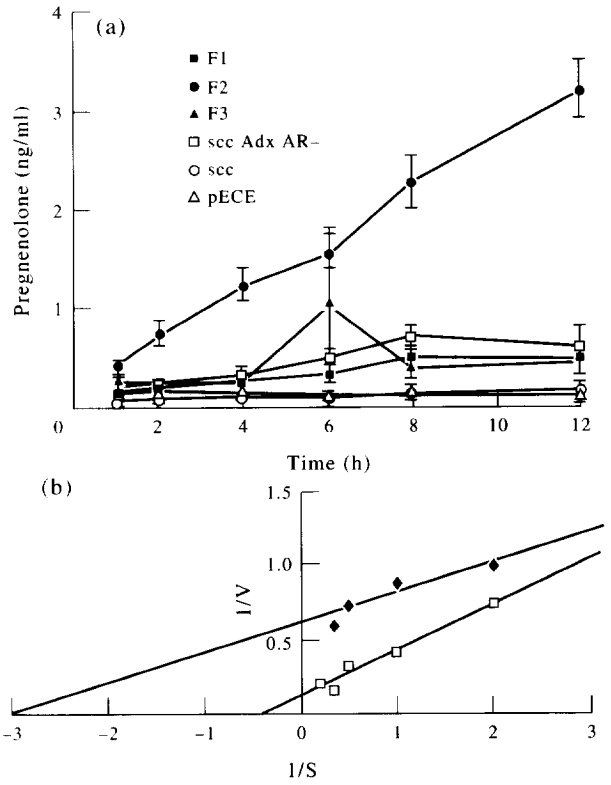

Fig. 2. Production of pregnenolone by transfected COS-1 cells. Cultures at about 60% confluence were transfected with plasmids in masses varied to yield amounts of *P*450scc sequences equivalent to 2 pmol of the vector expressing *P*450scc alone. (a) Time-course. Incubations with 5 μM 22-hydroxycholesterol were for the times shown, followed by immunoassay of pregnenolone. The data are from three independent transfections, each done with different plasmid preps and measured in triplicate. Pregnenolone values in ng/ml of culture medium are shown ±SEM. (b) Lineweaver–Burke analysis. Cells triply transfected with equimolar amounts of vectors expressing *P*450scc, Adx, and AdRed (◆) or transfected with an equimolar amount of vector expressing F2 (□) were incubated with 0.5–5.0 μM 22*R*-hydroxycholesterol. Data are averaged from three individual transfections, each done with different plasmid preps and assayed in triplicate. For further details see [57].

microsomal (Type II) *P*450 enzymes in place of the adrenodoxin/adrenodoxin reductase system used by mitochondrial (Type I) *P*450 enzymes.

Transfection of COS-1 cells with F6, a vector that directs the F2 fusion to the endoplasmic reticulum, results in no detectable conversion of 22-hydroxycholesterol to pregnenolone (Fig. 3). When a *P*450 oxidoreductase moiety is substituted for adrenodoxin reductase and adrenodoxin in the construct termed F4, some steriodogenesis persists in mitochondria, but when the F8 construct that directs this fusion protein to the endoplasmic reticulum is transfected, no steroidogenesis is detectable. Thus, in the mitochondria, *P*450scc can receive electrons effectively from a wide variety of donors, including adrenodoxin, adrenodoxin reductase, *P*450 oxidoreductase, and various fusions of these. However when directed to the endoplasmic reticulum neither the three individual components of the *P*450scc

system nor *P*450scc, plus *P*450 oxidoreductase nor any of the *P*450scc fusion constructions could produce any detectable pregnenolone. Thus the mitochondrial environment is absolutely mandatory for the enzymatic activity of *P*450scc.

THE ACUTE RESPONSE: MITOCHONDRIAL CHOLESTEROL TRANSPORT

As cholesterol can be converted to pregnenolone only in mitochondria, the transport of cholesterol from cytoplasmic stores into mitochondria is of paramount importance. Early work showed that cycloheximide and other inhibitors of protein synthesis can block ACTH-induced steroidogenesis in the adrenal or gonadotropin-induced steroidogenesis in ovarian granulosa cells [49, 67–69]. This has led to the search for various cycloheximide-sensitive "labile factors" that are required for the acute steroidogenic response. As discussed in the section on "The Chronic Response", it is now clear that such a factor is not a nuclear factor needed for the transcription of the genes for the steroidogenic enzymes. In fact most attention has been focussed on the transport of cholesterol into mitochondria; as we shall see below, this is indeed where the acute cycloheximide-sensitive regulator of adrenal and gonadal steroidogenesis lies.

Steroidogenic tissues can synthesize cholesterol directly from acetate [70], but prefer to use pre-formed cholesterol delivered to the cell as lipoproteins. Human steroidogenic cells receive most of their cholesterol from low density lipoproteins (LDL) while rodent tissues receive their cholesterol primarily from high-density lipoproteins (HDL) [71–73]. Human LDL carries cholesterol as cholesterol esters, which are taken up by receptor-mediated endocytosis and then stored in lipid droplets [72]. Cholesterol esters can also be produced from free cholesterol by cholesterol ester synthetase, and conversely free cholesterol is produced by the cholesterol ester hydrolase action of lysosomal acid cholesteryl ester hydrolase; mutations in this enzyme cause Wolman's Disease, a cholesterol ester storage disease affecting virtually all tissues [74]. Free cholesterol is insoluble in the aqueous cytosol, and must be transported to mitochondria in association with a protein.

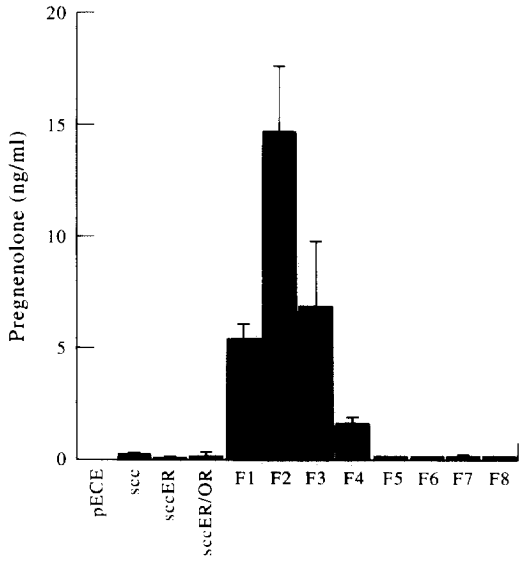

Fig. 3. Biological activity of the fusion proteins. Conversion of 22-hydroxycholesterol to pregnenolone was measured by RIA and is displayed as ng pregnenolone per mg of cellular protein. COS-1 cells were transfected with various expression vectors as designated in Fig. 1. For further details see [60].

The nature of the cytoplasmic cholesterol transport system is not completely clear, and several factors have been proposed to participate in this transport. It appears that Sterol Carrier Protein-2 (SCP-2) is an important factor [75–78]. Transfection of vectors expressing SCP-2 into cells will enhance steroidogenesis; SCP-2 can be induced by cAMP; and addition of SCP-2 to isolated mitochondria will enhance steroidogenesis [76], but it responds slowly to cAMP stimulation or inhibition by cycloheximide [79]. Thus SCP-2 appears to be a key component in targeting cholesterol to mitochondria, but is not the cycloheximide-sensitive "labile factor". Another protein called SCP has a very short half-life, is quite sensitive to cycloheximide, and shows a marked, ACTH-induced diurnal rhythm [80]. However, SCP functions primarily as a fatty acid-binding protein and not as a cholesterol-binding protein, and hence may not be involved in the acute steroidogenic response [78]. A 30-amino acid peptide termed Steroidogenesis Activator Peptide (SAP) has also received considerable attention. SAP is derived from the carboxy terminus of a 78 kDa protein termed glucose-regulated protein or BiP [81, 82]. SAP is found only in steroidogenic tissues; it has a short half-life and hence is very sensitive to cycloheximide, and it appears to facilitate the movement of cholesterol across the outer mitochondrial membrane [83, 84], but it does not appear to facilitate access of cholesterol to *P*450scc [53]. Thus the role of SAP remains uncertain. More recently substantial interest has been directed toward the mitochondrial benzodiazepine receptor (mBzR) and its endogenous ligand, termed diazepam-binding inhibitor (DBI) or endozepine. The mBzR appears to be composed of three subunits. An 18 kDa protein termed the peripheral benzodiazepine receptor (PBR) is associated with a 32 kDa voltage-dependent anion channel (VDAC) and a 30 kDa adenine nucleotide carrier. Both PBR and VDAC are found in the outer mitochondrial membrane, especially at "contact sites", where the outer and inner membranes are brought into contact, obliterating the aqueous intracisternal space [85]. By contrast, adenine nucleotide carrier is found on the inner mitochondrial membrane and may form a complex with PBR and VDAC [86], although this is somewhat controversial [87]. The endogenous ligand of this receptor complex is a 10 kDa peptide termed DBI or endozepine [88–90]. This protein was initially purified from brain [88, 89] but a derivative lacking 2

carboxy-terminal amino acids was also purified from bovine adrenal cells, where it facilitated the transport of cholesterol into mitochondria [91, 92]. There appears to be excellent evidence that the binding of endozepine to PBR will facilitate increased cholesterol transport into mitochondria and will increase steroidogenesis [87], but none of the components of this system appear to exhibit the exquisitely rapid response to cAMP and inhibition by cycloheximide that characterize the acute response.

A series of proteins generally termed pp30 is rapidly induced in adrenal and gonadal cells in response to tropic stimulators of steroidogenesis [93–95] and are transported to mitochondria [96, 97]. This protein appears as 37, 32 and 30 kDa forms in a precursor-product series and are rapidly phosphorylated in response to cAMP [96, 97]. This protein family is very rapidly induced by cAMP and inhibited by cycloheximide, they are localized to mitochondria and are found in the acutely regulated steroidogenic tissues: adrenal cortex, testicular Leydig cells and ovarian granulosa cells. The cDNA for this protein was recently cloned by Dr Douglas M. Stocco and colleagues from mouse MA-10 cells and was dubbed Steroidogenic Acute Regulatory Protein (StAR) [98]. Transfection of MA-10 cells with a vector expressing this protein increased conversion of cholesterol an average of 2.7-fold in the absence of hormonal stimulation [98], but this was on a background of StAR protein endogenously produced by the MA-10 cells. Thus this StAR protein is an excellent candidate for the mediator of the acute response.

One of the most effective ways of understanding the role of an anatomical structure or of a gene is to examine the effect on the organism when it is ablated. Conversely, genetic disease of unknown cause can be regarded as a genetic ablation experiment in which the responsible gene is unknown. In the past decade, virtually all of the genetically determined disorders of steroidogenesis have been solved. Virtually all have been disorders of the genes for the steroidogenic enzymes, which together comprise the various forms of congenital adrenal hyperplasia (for review see [1]), but this group also includes the gene for peroxisomal lignoceroyl-CoA ligase, that is disordered in adrenoleukodystrophy [99] and the DAX-1 gene disordered in congential adrenal hypoplasia [100]. The last remaining disorder of steroidogenesis to be solved, congential lipoid adrenal hyperplasia, (lipoid CAH) held the key to the acute response. In this rare disorder, the adrenals and gonads can make no steroids at all, and the adrenals become massively enlarge and engorged with cholesterol esters [101, 102]. The disorder is rare, with under 40 reported cases, but long-term survival is possible with early diagnosis and hormonal replacement therapy [102]. Mitochondria isolated from affected adrenal or gonadal tissue are unable to convert cholesterol to pregnenolone [102–104] so that the disease has been thought to be due to a disorder in *P*450scc [101–104]. It is particularly noteworthy that in 1972, Degenhart *et al.* showed that 22*R*-hydroxycholesterol, unlike cholesterol, could serve as substrate for steroidogenesis in affected mitochondria [103]. This result was interpreted as suggesting that the lesion in lipoid CAH was in a hypothetical 20α-hydroxylase. The demonstration that 20α- and 22-hydroxylase activity as well as C20–22 lyase activity all reside in one protein, *P*450scc, invalidated this conclusion, and Degenhart's work was largely forgotten. As we shall see below, this experiment actually demonstrated that the lesion in lipoid CAH was at a mitochondrial cholesterol transporter, but this was not understood until the recent, definitive work showing that the lesion in lipoid CAH was in a cholesterol transporter that could be circumvented by hydroxysterol substrate [105].

Early studies showed that the gene for *P*450scc was structurally intact in these patients [106] and more recently the sequencing of both the gene and cDNA from affected patients proved definitively that lipoid CAH was not due to a mutation in *P*450scc [107]. As P450scc was not involved and as the affected tissue accumulated large amounts of cholesterol ester, we postulated that lipoid CAH might be due to a mutation in a factor necessary to transport cholesterol to the mitochondria, i.e. a factor in the pathway distal to the cholesterol esterase that is involved in Wolman's disease and proximal to *P*450scc [107]. Northern blots of RNA from affected tissue of one patient showed that the sizes and abundances of the mRNAs for adrenodoxin, adrenodoxin reductase, SCP-2, GRP-78 (the precursor to SAP) and endozepine were all normal, suggesting that these factors were not responsible [107]. Cloning of PBR gene and cDNA from an affected individual ruled out the peripheral benzodiazepine receptor [108]. Thus the basis of lipoid CAH was unknown, but appeared to involve a cholesterol transporter of unknown nature.

Recently, we had the opportunity to evaluate two subsequent pregnancies in the woman who had borne the affected child in whom we had studied the genes for *P*450scc and PBR [107, 108]. By a combination of examining amniotic fluid steroids and examining the fetal genitalia by ultrasonography, we correctly diagnosed one pregnancy as unaffected and a later pregnancy as affected [109]. The fetal umbilical vein steroids in the affected fetus showed that there was no fetal adrenal or gonadal steroidogenesis as expected, but surprisingly showed that the placenta (a fetal tissue) still produced pregnenolone and progesterone [109]. This showed, for the first time, that the lesion in lipoid CAH did not affect all steroidogenic tissues, but instead was confined to the adrenals and gonads. Thus candidate genes could be more easily screened simply by examining their tissue distribution of expression. At about this time, the laboratory of Dr Jerome F. Strauss III cloned the human homologue of StAR and showed,

Table 1. Loss of StAR activity due to nonsense mutations

	Pregnenolone production (ng/dish)	
	F2	
Co-transfection	chol	20α
Vector	17 ± 3	60 ± 7
StAR	131 ± 23	60 ± 11
Patient 1	18 ± 5	56 ± 7
Patient 2 or 3	22 ± 4	75 ± 7

Using lipofectamine, nonsteroidogenic COS-1 monkey kidney cells were transfected with pSPORT (Vector) or with pSPORT expressing normal human StAR or patient mutants. The cells were co-transfected with the vector expressing fusion protein F2. The substrate was either the cellular and serum cholesterol (chol), or added (5 μg/ml) 20α-hydroxycholesterol (20α). After 48 h of incubation, the medium was collected and assayed for pregnenolone by immunoassays. Values are the means ± standard deviations from four separate transfections. For further details see [105].

for the first time, that StAR was expressed in the adrenals and gonads, but not in the placenta or brain [110]. Thus, in collaboration with Drs Strauss and Stocco, we examined the StAR cDNA and gene in three patients affected with lipoid CAH, and found that each was homozygous for a premature translation-termination (stop) codon, that disrupted StAR synthesis [105]. Thus StAR, a candidate factor for the acute response, was the basis of absent steroidogenesis in the adrenals and gonads, the tissues that exhibit the acute steroidogenic response, but was not expressed in normal placenta or brain, two steroidogenic tissues which apparently do not exhibit an acute response.

The definitive proof that StAR is the mitochondrial cholesterol transporter that mediates the acute response, however, comes from transfection experiments. We used non-steroidogenic COS-1 cells, so that we could examine the effects of StAR in the absence of a background of endogenous StAR expression. The COS-1 cells were either doubly transfected with vectors expressing *P*450scc and adrenodoxin, or transfected with the vector expressing the F2 construct [57] described above. Incubation of these COS-1 cells with cholesterol yielded no steroidogenesis unless they were transfected with the vector expressing wild-type, but not mutant, StAR. More importantly, all of the cell transfectants with or without normal or mutant StAR, could produce equivalent amounts of pregnenolone when given 20α-hydroxycholesterol as substrate rather than cholesterol [105] (Table 1). This experiment in whole cells effectively repeats Degenhart's experiment with isolated affected mitochondria, but now yields a different interpretation. The requirement for StAR was for access of the cholesterol to mitochondria, a transport process that can be circumvented by polar hydroxysterols such as 20α-hydroxycholesterol or the 22*R*-cholesterol used in our fusion protein studies above [57, 60]. Thus the studies with lipoid CAH definitively establish StAR as the factor that is responsible for the acute steroidogenic response.

The human StAR gene has now been cloned [111], additional StAR mutations causing lipoid CAH have been described [112], and the hormonal and developmental regulation of mouse StAR have been characterized [113] further confirming StAR's role in steroidogenesis. However, the mechanism of StAR action remains unclear. The StAR precursor is rapidly synthesized in the cytoplasm in response to cAMP, and thence undergoes two proteolytic cleavage events as it traverses the mitochondrial membranes. Once inside the mitochondria, it is degraded rapidly. Thus the function of StAR may simply be to traverse the mitochondrial membranes. In doing so, it may function by generating contact sites between the outer and inner mitochondrial membranes, thus facilitating the flow of cholesterol from the outer to the inner membrane [98]. This would be consistent with the observation that the mitochondria have two pools of cholesterol, one which is stable and cannot be used as substrate for steroidogenesis, and the other which is labile, and becomes the steroidogenic substrate [114]. Much remains to be learned about StAR and the acute response, and roles for SCP-2, PBR, and SAP have not been excluded. The field of intracellular and intra-mitochondrial cholesterol transport promises to be one of the most exciting areas of research in steroidogenesis in the next several years.

Acknowledgements—I gratefully acknowledge the contributions of Drs Jennifer Harikrishna and Stephen M. Black, who conducted the fusion protein experiments, and Dr Dong Lin, who conducted the genetic experiments with lipoid CAH. This work was supported by a Glaxo Cardiovascular Discovery Grant, NIH Grants DK 37922 and DK 42154, and March of Dimes Grant 6-396, to WLM, and by the Child Health Research Center Grant (HD 28825) to the Dept of Pediatrics, University of California, San Francisco. I also thank my collaborators Dr Jerome F. Strauss III, University of Pennsylvania, whose laboratory performed the functional assays with StAR and Dr Douglas M. Stocco, Texas Tech University, whose laboratory first cloned StAR from mouse cells.

REFERENCES

1. Miller W. L. and Tyrrell J. B.: The adrenal cortex. In *Endocrinology and Metabolism* (Edited by P. Felig, J. Baxter and L. Frohman). McGraw Hill, NY (1995) pp. 555–711.
2. Voutilainen R. and Miller W. L.: Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain cleavage enzyme, P450scc, in human steroidogenic tissues. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 1590–1594.
3. Mesiano S., Mellon S. H., Gospodarowicz D., Di Blasio A. M. and Jaffe R. B.: Basic fibroblast growth factor expression is regulated by corticotropin in the human fetal adrenal: a model for adrenal growth regulation. *Proc. Natn. Acad. Sci. U.S.A.* **88** (1991) 5428–5432.

4. Mesiano S., Mellon S. H. and Jaffe R. B.: Mitogenic action, regulation, and localization of insulin-like growth factors in the human fetal adrenal gland. *J. Clin. Endocr. Metab.* **76** (1993) 968–976.
5. Townsend S., Dallman M. F. and Miller W. L.: Rat insulin-like growth factors-I and -II mRNAs are unchanged during compensatory adrenal growth but decrease during ACTH-induced adrenal growth. *J. Biol. Chem.* **265** (1990) 22,117–22,122.
6. Simpson E. R.: Cholesterol side-chain cleavage, cytochrome *P*450, and the control of steroidogenesis. *Molec. Cell. Endocr.* **13** (1979) 213–227.
7. Kimura T.: ACTH stimulation on cholesterol side chain cleavage activity of adrenocortical mitochondria. *Molec. Cell. Biochem.* **36** (1981) 105–122.
8. Hall P. F.: Tropic stimulation of steroidogenesis: In serach of the elusive trigger. *Rec. Prog. Horm. Res.* **41** (1985) 1–39.
9. Miller W. L.: Molecular biology of steroid hormone synthesis. *Endocrine Rev.* **9** (1988) 295–318.
10. Simpson E. R. and Waterman M. R.: Regulation of synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Ann. Rev. Physiol.* **50** (1988) 427–440.
11. Lieberman S. and Prasad V. V. K.: Heterodox notions on pathways of steroidogenesis. *Endocrine Rev.* **11** (1990) 469–493.
12. Lambeth J. D. and Pember S. O.: Cytochrome *P*-450scc-adrenodoxin complex: reduction properties of the substrate-associated cytochrome and relation of the reduction states of heme and iron–sulfur centers to association of the proteins. *J. Biol. Chem.* **258** (1983) 5596–5602.
13. Voutilainen R., Tapanainen J., Chung B., Matteson K. J. and Miller W. L.: Hormonal regulation of *P*450scc (20,22-desmolase) and *P*450c17 (17α-hydroxylase/17,20-lyase) in cultured human granulosa cells. *J. Clin. Endocr. Metab.* **63** (1986) 202–207.
14. Mellon S. H. and Deschepper C. F.: Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. *Brain Res.* **629** (1993) 283–292.
15. Voutilainen R., Picado-Leonard J., DiBlasio A. M. and Miller W. L.: Hormonal and developmental regulation of human adrenodoxin mRNA in steroidogenic tissues. *J. Clin. Endocr. Metab.* **66** (1988) 383–388.
16. Picado-Leonard J., Voutilainen R., Kao L., Chung B., Strauss J. F. III and Miller W. L.: Human adrenodoxin: cloning of three cDNAs and cycloheximide enhancement in JEG-3 cells. *J. Biol. Chem.* **263** (1988) 3240–3244, corrected 11016.
17. Brentano S. T., Black S. M., Lin D. and Miller W. L.: cAMP post-transcriptionally diminishes the abundance of adrenodoxin reductase mRNA. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 4099–4103.
18. John M. E., John M. C., Boggaram V., Simpson E. R. and Waterman M. R.: Transcriptional regulation of steroid hydroxylase genes by corticotropin. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 4715–4719.
19. Golos T. J., Miller W. L. and Strauss J. F. III: Human chorionic gondadotropin and 8-bromo-cyclic adenosine mono phosphate promote an acute increase in cytochrome *P*450scc and adrenodoxin messenger RNAs in cultured human granulosa cells by a cycloheximide-insensitive mechanism. *J. Clin. Invest.* **80** (1987) 896–899.
20. Ringler G. E., Kao L., Miller W. L. and Strauss J. F. III: Effects of 8-bromo-cAMP on expression of endocrine functions by cultured human trophoblast cells. Regulation of specific mRNAs. *Molec. Cell. Endocr.* **62** (1989) 13–21.
21. Mellon S. H. and Vaisse C.: cAMP regulates *P*450scc gene expression by a cycloheximide-insensitive mechanism in cultured mouse leydig MA-10 cells. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 7775–7779.
22. Mellon S. H., Kushner J. A. and Vaisse C.: Expression and regulation of adrenodoxin and *P*450scc mRNAs in rodent tissues. *DNA Cell Biol.* **10** (1991) 339–347.
23. Rice D. A., Mouw A. R., Bogerd A. M. and Parker K. L.: A shared promoter element regulates the expression of three steroidogenic enzymes. *Molec. Endocr.* **5** (1991) 1552–1561.
24. Lala D. S., Rice D. A. and Parker K. L.: Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of *fushi-tarazu* factor I. *Molec. Endocr.* **6** (1992) 1249–1258.
25. Morohashi K., Honda S., Inomata Y., Handa H. and Omura T.: A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic *P*-450s. *J. Biol. Chem.* **267** (1992) 17,913–17,919.
26. Honda S., Morohashi K., Nmura M., Takeya M., Kitajima M. and Omura T.: Ad4BP regulating steroidogenic *P*-450 genes is a member of steroid hormone receptor superfamily. *J. Biol. Chem.* **268** (1993) 7479–7502.
27. Guo I., Huang C. and Chung B.: Differential regulation of the CYP11A1 (*P*450scc) and ferredoxin genes in adrenal and placental cells. *DNA Cell Biol.* **12** (1993) 849–860.
28. Guo I., Tsai H. and Chung B.: Actions of two different cAMP-responsive sequences and an enhancer of the human *CYP11A1* (*P*450scc) gene in adrenal Y1 and placental JEG-3 cells. *J. Biol. Chem.* **269** (1994) 6362–6369.
29. Moore C. C. D. and Miller W. L.: The role of transcriptional regulation in steroid hormone biosynthesis. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 517–525.
30. Hum D. W. and Miller W. L.: Transcriptional regulation of human genes for steroidogenic enzymes. *Clin. Chem.* **39** (1993) 333–340.
31. Black S. M., Szklarz G. D., Harikrishna J. A., Lin D., Wolf C. R. and Miller W. L.: Regulation of proteins of the cholesterol side-chain cleavage system in Y-1 and JEG-3 cells. *Endocrinology* **132** (1993) 539–545.
32. Inoue H., Higashi Y., Morohashi K. and Fujii-Kuriyama Y.: The 5′ flanking region of the human *P*-450(SCC) gene shows responsiveness to cAMP-dependent regulation in a transient gene-expression system of Y-1 adrenal tumor cells. *Eur. J. Biochem.* **171** (1988) 435–440.
33. Ahlgren R., Simpson E. R., Waterman M. R. and Lund J.: Characterization of the promoter/regulatory region of the bovine CYP11A (*P*450scc) gene. *J. Biol. Chem.* **265** (1990) 3313–3319.
34. Moore C. C. D., Brentano S. T. and Miller W. L.: Human *P*450scc gene transcription is induced by cyclic AMP and repressed by 12-*O*-tetradecanolyphorbol-13-acetate and A23187 by independent *cis*-elements. *Molec. Cell. Biol.* **10** (1990) 6013–6023.
35. Inoue H., Watanabe N., Higashi Y. and Fujii-Kuriyama Y.: Structures of regulatory regions in the human cytochrome *P*-450scc (desmolase) gene. *Eur. J. Biochem.* **195** (1991) 563–569.
36. Moore C. C. D., Hum D. W. and Miller W. L.: Identification of positive and negative placental-specific basal elements, a transcriptional repressor, and a cAMP response element in the human gene for *P*450scc. *Molec. Endocr.* **6** (1992) 2045–2058.
37. Brentano S. T. and Miller W. L.: Regulation of human *P*450scc and adrenodoxin mRNAs in JEG-3 cytotrophoblast cells. *Endocrinology* **131** (1992) 3010–3018.
38. Begeot M., Shetty U., Kilgore M., Waterman M. and Simpson E.: Regulation of expression of the CYP11A (*P*450scc) gene in bovine ovarian luteal cells by forskolin and phorbol esters. *J. Biol. Chem.* **268** (1993) 17,317–17,325.
39. Hum D. W., Staels B., Black S. M. and Miller W. L.: Basal transcriptional activity and cyclic AMP responsiveness of the human *P*450scc promotor transfected into mouse Leydig MA-10 cells. *Endocringology* **132** (1993) 546–552.
40. Hum D. W., Aza-Blanc P. and Miller W. L.: Characterization of placental transcriptional activation of the human gene for *P*450scc. *DNA Cell Biol.* 14 (1995) 451–463.
41. Dahl E. V. and Bahn R. C.: Aberrant adrenal cortical tissue near the testis in human infants. *Am. J. Path.* **40** (1962) 587–598.
42. Stone D. and Hechter O.: Studies on ACTH action in perfused bovine adrenals: aspects of progesterone as an intermediary in cortico-steroidogenesis. *Arch. Biochem. Biophys.* **54** (1955) 121–128.
43. Kortiz S. B. and Kumar A. M.: On the mechanism of action of adrenocorticotropic hormone—the stimulation of the activity of enzymes involved in pregnenolone synthesis. *J. Biol. Chem.* **245** (1970) 152–157.
44. Jefcoate C. R., Simpson E. R. and Boyd G. S.: Spectral properties of rat adrenal-mitochondrial cytochrome *P*-450. *Eur. J. Biochem.* **42** (1974) 539–551.
45. Kramer R. E., Gallant S. and Brownie A. C.: Actions of angiotensin II on aldosterone biosynthesis in the rat adrenal cortex. *J. Biol. Chem.* **255** (1980) 3442–3447.
46. Kramer R. E., Rainey W. E., Funkenstein B., Dee A., Simpson E. R. and Waterman M. R.: Induction of synthesis of

mitochondrial steroidogenic enzymes of bovine adrenocortical cells by analogs of cyclic AMP. *J. Biol. chem.* **259** (1984) 707–713.
47. DiBlasio A. M., Voutilainen R., Jaffe R. B. and Miller W. L.: Hormonal regulation of mRNAs for *P*450scc (cholesterol side-chain cleavage enzyme) and *P*450c17 (17α-hydroxylase/17,20 lyase) in cultured human fetal adrenal cells. *J. Clin. Endocr. Metab.* **65** (1987) 170–175.
48. Morisaki M., Duque C., Ikekawa H. and Shikita M.: Substrate specificity of adrenocortical cytochrome *P*450. I. Effect of structural modification of cholesterol side chain on pregnenolone production. *J. Steroid Biochem.* **13** (1980) 545–550.
49. Ferguson J. J.: Protein synthesis and adrenocorticotropin responsiveness. *J. Biol. Chem.* **238** (1963) 2754–2759.
50. Garren L. D., Ney R. L. and Davis W. W.: Studies on the role of protein synthesis in the regulation of corticosterone production by adrenocorticotropic hormone *in vivo*. *Proc. Natn. Acad. Sci. U.S.A.* **53** (1965) 1443–1450.
51. Davis W. W. and Garren L. D.: On the mechanism of action of adrenocorticotropic hormone. *J. Biol. Chem.* **243** (1968) 5153–5157.
52. Cooke B. A., Janszen F. H. A., Clotscher W. F. and van der Molen H. J.: Effect of protein-synthesis inhibitors on testosterone production in rat testis interstitial tissue and Leydig cell preparations. *Biochem. J.* **150** (1975) 413–418.
53. Privalle C. T., Crivello J. F. and Jefcoate C. R.: Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome *P*-450 in rat adrenal gland. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 702–706.
54. Jefcoate C. R., McNamara B. C. and DiBartolomeis M. S.: Control of steroid synthesis in adrenal fasciculata. *Endocrine Res.* **12** (1986) 315–350.
55. Hanukoglu I., Suh B. S., Himmelhoch S. and Amsterdam A.: Induction and mitochondrial localization of cytochrome *P*450scc system enzymes in normal and tranformed ovarian granulosa cells. *J. Cell Biol.* **111** (1990) 1373–1381.
56. Zuber M. X., Mason J. I., Simpson E. R. and Waterman M. R.: Simultaneous transfection of COS-1 cells with mitochondrial and microsomal steroid hydroxylases: incorporation of a steroidogenic pathway into non-steroidogenic cells. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 699–703.
57. Harikrishna J. A., Black S. M., Szklarz G. D. and Miller W. L.: Construction and function of fusion enzymes of the human cytochrome *P*450scc system. *DNA Cell Biol.* **12** (1993) 371–379.
58. Coghlan V. M. and Vickery L. E.: Site-specific mutations in human ferredoxin that affect binding to ferredoxin reductase and cytochrome *P*450scc. *J. Biol. Chem.* **266** (1991) 18,606–18,612.
59. Coghlan V. M. and Vickery L. E.: Electrostatic interactions stabilizing ferredoxin electron transfer complexes: disruption by "conservative" mutations. *J. Biol. Chem.* **267** (1992) 8932–8935.
60. Black S. M., Harikrishna J. A., Szklarz G. D. and Miller W. L.:The mitochondrial environment is required for activity of the cholesterol side-chain cleavage enzyme, cytochrome *P*450scc. *Proc. Natn. Acad. Sci. U.S.A.* **91** (1994) 7247–7251.
61. Nahri L. O. and Fulco A. J.: Characterization of a catalytically self-sufficient 119,000–dalton *P*450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **261** (1986) 7160–7169.
62. Nahri L. O. and Fulco A. J.: Identification and characterization of two functional domains in cytochrome *P*450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **262** (1987) 6683–6690.
63. Ruettinger R. T., Wen L. P. and Fulco A. J.: Coding nucleotide, 5′ regulatory and deduced amino acid sequences of *P*450BM-3, a single peptide cytochrome *P*450: NADPH-*P*450 reductase from *Bacillus megaterium*. *J. Biol. Chem.* **264** (1989) 10,987–10,995.
64. Sakaki T., Shibata M., Yabusaki Y., Murakami H. and Ohkawa H.: Expression of bovine cytochrome *P*450c21 and its fused enzymes with yeast NADPH-cytochrome *P*450 reductase in *Saccaromyces cerevisiae*. *DNA Cell Biol.* **9** (1990) 603–614.
65. Sakaki T., Akiyoshi-Shibata M., Yabusaki Y. and Ohkawa H.: Organella-targeted expression of rat liver cytochrome *P*450c27 in yeast. Genetically engineered alteration of mitochondrial *P*450 into a microsomal form creates a novel function electron transport chain. *J. Biol. Chem.* **267** (1992) 16,497–16,502.
66. Toaff M. E., Schleyer H. and Strauss J. F. I.: Metabolism of 25-hydroxycholesterol by rat luteal mitochondria and dispersed cells. *Endocrinology* **111** (1982) 1785–1790.
67. Garren L. D., Ney R. L. and Davis W. W.: Studies on the role of protein synthesis in the regulation of corticosterone production by ACTH *in vivo*. *Proc. Natn. Acad. Sci. U.S.A.* **53** (1965) 1443–1450.
68. Simpson E. R., McCarthy J. L. and Peterson J. A.: Evidence that the cycloheximide-sensitive site of adrenocorticotropic hormone action is in the mitochondrion. *J. Biol. Chem.* **253** (1978) 3135–3139.
69. Toaff M. E., Strauss J. F. III, Flickinger G. L. and Shattil S. J.: Relationship of cholesterol supply to luteal mitochondrial steroid synthesis. *J. Biol. Chem.* **254** (1979) 3977–3982.
70. Hechter O., Solomon M. M., Zaffaroni A. and Pincus G.: Transformation of cholesterol and acetate to adrenal cortical hormones. *Arch. Biochem. Biophys.* **46** (1953) 201.
71. Andersen J. M. and Dietschy J. M.: Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary and testis of the rat. *J. Biol. Chem.* **253** (1978) 9024.
72. Brown M. S., Kovanen P. T. and Goldstein J. L.: Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for steroid synthesis in the adrenal cortex. *Rec. Prog. Horm. Res.* **35** (1979) 215–257.
73. Gwynne J. T. and Strauss J. F. III: The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocrine Rev.* **3** (1982) 299–329.
74. Anderson R. A., Byrum R. S., Coates P. M. and Sando G. N.: Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease. *Proc. Natn. Acad. Sci. U.S.A.* **91** (1994) 2718–2722.
75. Noland B. J., Arebalo R. E., Hansbury E. and Scallen T. J.: Purification and properties of sterol carrier protein. *J. Biol. Chem.* **255** (1980) 4282–4289.
76. Chandebhan R., Noland B. J., Scallen T. J. and Vahouny G. V.: Sterol carrier protein 2: delivery of cholesterol from adrenal lipid droplets to mitochondria for pregnenolone synthesis. *J. Biol. Chem.* **257** (1982) 8928–8934.
77. Vahouny G. V., Chanderbhan R., Noland B. J., Irwin D., Dennis P., Labmeth J. D. and Scallen T. J.: Sterol carrier protein 2: identification of adrenal sterol carrier protein 2 and site of action for mitochondrial cholesterol utilization. *J. Biol. Chem.* **258** (1983) 11,731–11,737.
78. Scallen T. J., Noland B. J., Gavey K. L., Bass N. M., Ockner R. K., Chanderbhan R. and Vahouny G. V.: Sterol carrier protein 2 and fatty acid-binding protein: separate and distinct physiological functions. *J. Biol. Chem.* **260** (1985) 4733–4739.
79. van Amerongen A., Demel R. A., Westerman J. and Wirtz K.W. A.: Transfer of cholesterol and oxysterol derivatives by the nonspecific lipid transfer protein (sterol carrier protein 2): a study on its mode of action. *Biochim. Biophys. Acta* **1004** (1989) 36–43.
80. Conneely O. M., Headon D. R., Olson C. D., Ungar F. and Dempsey M. E.: Intra-mitochondrial movement of adrenal sterol carrier protein with cholesterol in response to corticotropin. *Proc. Natn. Acad. Sci. U.S.A.* **81** (1984) 2970.
81. Ting J. and Lee A. S.: Human gene encoding the 78,000-dalton glucose-regulated protein and its pseudogene: structure, conservation, and regulation. *DNA* **7** (1988) 275–286.
82. Li X., Warren D. W., Gregorie J., Pedersen R. C. and Lee A. S.: The rat 78,000 dalton glucose regulated protein (GRP-78) as a precursor for the rat steroidogenesis—activator polypeptide (SAP): the SAP coding sequence is homologous with the terminal end of GRP-78. *Molec. Endocr.* **3** (1989) 1944–1952.
83. Pedersen R. C. and Brownie A. C.: Cholesterol side-chain cleavage in the rat adrenal cortex: isolation of a cycloheximide-sensitive activator peptide. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 1882–1886.
84. Pedersen R. C. and Brownie A. C.: Steroidogenesis activator polypeptide isolated from a rat Leydig cell tumor. *Science* **236** (1987) 188–190.
85. Levitt D.: Gramicidin, VDAC, porin, and perforin channels. *Curr. Opin. Cell. Biol.* **2** (1990) 689–694.

86. McEnery M. W., Snowman A. M., Trifiletti R. R. and Snyder S. H.: Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 3170–3174.
87. Papadopoulos V.: Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. *Endocrine Rev.* **14** (1993) 222–240.
88. Guidotti A., Forchetti C. M., Corda M. G., Konkel D., Bennet C. D. and Costa E.: Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 3531–3533.
89. Shoyab M., Gentry L. E., Marquardt H. and Todaro G.: Isolation and characterization of a putative endogenous benzodiazepinoid (Endozepine) from bovine and human brain. *J. Biol. Chem.* **261** (1986) 11,968–11,973.
90. Todaro G. J., Rose T. M. and Shoyab M.: Human DBI (endozepine): relationship to the homologous membrane-associated protein. *Neuropharmacology* **30** (1991) 1373–1380.
91. Yanagibashi K., Ohno Y., Kawamura M. and Hall P. F.: The regulation of intracellular transport of cholesterol in bovine adrenal cells: purification of a novel protein. *Endocrinology* **123** (1988) 2075–2082.
92. Besman M. J., Yanagibashi K., Lee T. D., Kawamura M., Hall P. F. and Shively J. E.: Identification of des-(Gly-Ile)-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 4897–4901.
93. Krueger R. J. and Orme-Johnson N. R.: Acute adrenocorticotropic hormone stimulation of adrenal corticosteroidogenesis. *J. Biol. Chem.* **258** (1983) 10,159–10,167.
94. Pon L. A. and Orme-Johnson N. R.: Acute stimulation of steroidogenesis in corpus luteum and adrenal cortex by peptide hormones. *J. Biol. Chem.* **261** (1986) 6594–6599.
95. Stocco D. M. and Kilgore M. W.: Induction of mitochondrial proteins in MA-10 Leydig tumour cells with human chorigonadotropin. *Biochem. J.* **249** (1988) 95–103.
96. Stocco D. M. and Sodeman T. C.: The 30 kDa mitochondrial protein induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors. *J. Biol. Chem.* **266** (1991) 19,731–19,738.
97. Epstein L. F. and Orme-Johnson N. R.: Regulation of steroid hormone biosynthesis. Identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells. *J. Biol. Chem.* **266** (1991) 19,739–19,745.
98. Clark B. J., Wells J., King S. R. and Stocco D. M.: The purification cloning and expression of a novel luteinizing hormone-induced mitochondrial protein in mouse Leydig MA-10 cells. *J. Biol. Chem.* **269** (1994) 28,314–28,322.
99. Mosser J., Douar A. M., Sarde C. O., Kioschis P., Feil R., Moser H., Poustka A. M., Mandel J. M. and Aubourg P.: Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* **361** (1993) 726–730.
100. Zanaria E., Muscatelli F., Bardoni B., Strom T. M., Guioli S., Guo W., Lalli E., Moser C., Walker A. P., McCabe E. R. B., Meitinger T., Monaco A. P., Sassone-Corsi P. and Camerino G.: An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature* **372** (1994) 635–641.
101. Camacho A. M., Kowarski A., Migeon C. J. and Brough A.: Congenital adrenal hyperplasia due to a deficiency of one of the enzymes involved in the biosynthesis of pregnenolone. *J. Clin. Endocr. Metab.* **28** (1968) 153–161.
102. Hauffa B. P., Miller W. L., Grumbach M. M., Conte F. A. and Kaplan S. L.: Congenital adrenal hyperplasia due to deficient cholesterol side-chain cleavage activity (20,22 desmolase) in a patient treated for 18 years. *Clin. Endocr.* **23** (1985) 481–493.
103. Degenhart H. J., Visser K. H. A., Boon H. and O'Doherty N. J. D.: Evidence for deficiency of 20α cholesterol hydroxylase activity in adrenal tissue of a patient with lipoid adrenal hyperplasia. *Acta Endocr.* **71** (1972) 512–518.
104. Koizumi S., Kyoya S., Miyawaki T., Kidani H., Funabashi T., Nakashima H., Nakanuma Y., Ohta G., Itagaki E. and Katagiri M.: Cholesterol side-chain cleavage enzyme activity and cytochrome *P*450 content in adrenal mitochondria of a patient with congenital lipoid adrenal hyperplasia (Prader disease). *Clin. Chem. Acta* 77 (1977) 301–306.
105. Lin D., Sugawara T., Strauss J. F. III, Clark B. J., Stocco D. M., Saenger P., Rogol A. and Miller W. L.: Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** (1995) 1828–1831.
106. Matteson K. J., Chung B., Urdea M. S. and Miller W. L.: Study of cholesterol side chain cleavage (20,22 desmolase) deficiency causing congenital lipoid adrenal hyperplasia using bovine-sequence *P*450scc oligodeoxyribonucleotide probes. *Endocrinology* **118** (1986) 1296–1305.
107. Lin D., Gitelman S. E., Saenger P. and Miller W. L.: Normal genes for the cholesterol side chain cleavage enzyme, *P*450scc, in congenital lipoid adrenal hyperplasia. *J. Clin. Invest.* **88** (1991) 1955–1962.
108. Lin D., Chang Y. J., Strauss J. F. III and Miller W. L.: The human peripheral benzodiazepine receptor gene. Cloning and characterization of alternative splicing in normal tissues and in a patient with congenital lipoid adrenal hyperplasia. *Genomics* **18** (1993) 643–650.
109. Saenger P., Klonari Z., Black S. M., Compagnone N., Mellon S. H., Fleischer A., Abrams C. A. L., Shackleton C. H. L. and Miller W. L.: Prenatal diagnosis of congenital lipoid adrenal hyperplasia. *J. Clin. Endocr. Metab.* **80** (1995) 200–205.
110. Sugawara T., Holt J. A., Driscoll D., Strauss J. F. III, Lin D., Miller W. L., Patterson D., Clancy K. P., Hart I. M., Clark B. J. and Stocco D. M.: Human steroidogenic acute regulatory protein (StAR): functional activity in COS cells, tissue-expression, mapping of the structural gene to 8p11.2 and an expressed pseudogene to chromosome 13. *Proc. Natn. Acad. Sci. U.S.A.* **92** (1995) 4778–4782.
111. Sugawara T., Lin D., Holt J. A., Martin K. O., Javitt N. B., Miller W. L. and Strauss J. F. III: The structure of the human steroidogenic acute regulatory (StAR) protein gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. *Biochemistry* **34** (1995) 12,506–12,512.
112. Tee M. K., Sugawara T., Holt J. A., Guiguen Y., Buckingham B., Strauss J. F. III and Miller W. L.: T→A transversion 11 bp from a splice acceptor site in the gene for steroidogenic acute regulatory protein causes congenital lipoid adrenal hyperplasia. *Hum. Molec. Genet.* **4** (1995) 2299–2305.
113. Clark B. J., Soo S. C., Caron K. M., Ikeda Y., Parker K. L. and Stocco D. M.: Hormonal and developmental regulation of the steroidogenic acute regulatory protein. *Molec. Endocr.* **9** (1995) 1346–1355.
114. Stevens V. L., Xu T. and Lambeth J. D.: Cholesterol pools in rat adrenal mitochondria: use of cholesterol oxidase to infer a complex pool structure. *Endocrinology* **130** (1992) 1557–1563.