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MINIREVIEW

StAR Search—What We Know about How the Steroidogenic Acute Regulatory Protein Mediates Mitochondrial Cholesterol Import

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Cholesterol is the starting point for biosynthesis of steroids, oxysterols and bile acids, and is also an essential component of cellular membranes. The mechanisms directing the intracellular trafficking of this insoluble molecule have received attention through the discovery of the steroidogenic acute regulatory protein (StAR) and related proteins containing StAR-related lipid transfer domains. Much of our understanding of the physiology of StAR derives from studies of congenital lipoid adrenal hyperplasia, which is caused by StAR mutations. Multiple lines of evidence show that StAR moves cholesterol from the outer to inner mitochondrial membrane, but acts exclusively on the outer membrane. The precise mechanism by which StAR's action on the outer mitochondrial membrane stimulates the flow of cholesterol to the inner membrane remains unclear. When StAR interacts with protonated phospholipid head groups on the outer mitochondrial membrane, it undergoes a conformational change (molten globule transition) that opens and closes StAR's cholesterol-binding pocket; this conformational change is required for cholesterol binding, which is required for StAR activity. The action of StAR probably requires interaction with the peripheral benzodiazepine receptor. (*Molecular Endocrinology* 21: 589–601, 2007)

THE MECHANISMS REGULATING the cellular distribution of cholesterol to intracellular targets is of particular interest because cholesterol is the starting material for the synthesis of steroid hormones, oxysterols, and vitamin D. Cholesterol is also an essential component of intracellular and plasma membranes, being required for membrane fluidity, and hence for cellular motion. Vertebrates can also add cholesterol to the carboxyl termini of hedgehog proteins, which direct multiple aspects of early embryonic development, whereas steroids and sterols derived from cholesterol activate a broad spectrum of nuclear and membrane-based receptors. Thus cholesterol metabolism is of central interest to the study of development. Recent work with the structural and cellular biology of the steroidogenic acute regulatory protein (StAR) and structurally related proteins has illuminated the processes regulating steroidogenesis and revealed novel mechanisms of intracellular cholesterol distribution.

CHOLESTEROL TRAFFICKING

Most cells can synthesize cholesterol *de novo* from acetyl coenzyme A and can also take up cholesterol from circulating lipoprotein particles; human cells typically utilize receptors for low density lipoproteins, whereas rodent cells utilize scavenger receptor BI (1). The intracellular cholesterol economy is largely regulated by the SREBPs (sterol response element binding proteins) group of transcription factors, which regulate genes involved in the synthesis of cholesterol and fatty acids (2). Genetic disorders of cholesterol biosynthesis, such as the Smith-Laemli-Opitz syndrome caused by mutation of cholesterol 7-dehydrogenase, are well described (3). Cholesterol can be esterified by acyl-coenzyme A cholesterol transferase, stored in lipid droplets, and accessed for cellular needs by activation of cholesterol esterase (hormone-sensitive lipase) (Fig. 1). Once liberated as free cholesterol, the cell faces the

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Abbreviations: CAH, Congenital adrenal hyperplasia; CD, circular dichroism; CRAC, cholesterol recognition amino acid consensus; IMM, inner mitochondrial membrane; IMS, intramembranous space; MD, molecular dynamics; MLN64, metastatic lymph node, clone 64; OMM, outer mitochondrial membrane; PBR, peripheral benzodiazepine receptor; SREBP, sterol response element-binding protein; StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transfer; SUV, small unilamellar vesicle; VDAC, voltage-dependent anion channel.

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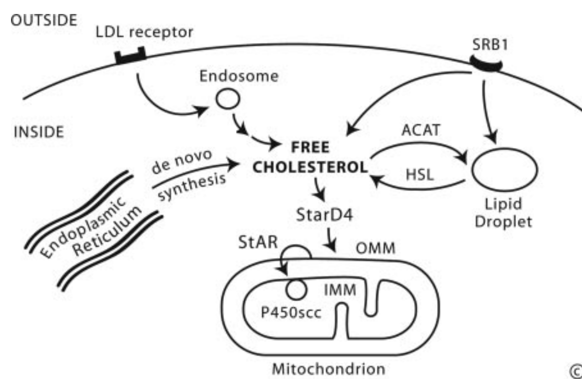

Fig. 1. Principal Features of the Cellular Cholesterol Economy

Human cells typically pick up circulating low-density lipoproteins (LDL) through receptor-mediated endocytosis, directing the cholesterol to endosomes. Rodent cells pick up high-density lipoproteins (HDL) via scavenger receptor B1 (SRB1) and direct it to lipid droplets. Cholesterol can also be synthesized *de novo* from acetate in the endoplasmic reticulum. Irrespective of source, cholesterol can be esterified by acyl-CoA: cholesterol transferase (ACAT) and stored in lipid droplets as cholesterol esters. Free cholesterol, produced by the action of hormone-sensitive lipase (HSL), is probably bound by StarD4 for trans-cytoplasmic transport to membrane destinations, including the OMM. In the adrenals and gonads, StAR is responsible for the rapid movement of cholesterol from the OMM to the IMM, where it can be taken up by the cholesterol side-chain cleavage enzyme, P450scc, and converted to pregnenolone.

novel problem of transporting this nearly insoluble molecule to various cellular membranes, distributing it appropriately, and inserting it into a membrane. Steroidogenic cells face the additional requirement to deliver large amounts of cholesterol to the cholesterol side-chain cleavage enzyme, which lies on the inner mitochondrial membrane (IMM) where it is the first and rate-limiting enzyme in steroidogenesis, converting insoluble cholesterol to soluble pregnenolone (4).

Because cholesterol is essentially insoluble, cells have devised two ways to deliver cholesterol across aqueous cytoplasmic spaces. First, cholesterol can be incorporated into the membranes of vesicles (lysosomes, endosomes, peroxisomes), which can then fuse with other membranes, thus delivering cholesterol from one intracellular compartment to another; this appears to be a minor pathway (5). Second, cholesterol can be rendered soluble by binding to proteins. Early work focused on sterol-carrier protein 2 and its homolog sterol-carrier protein x, but these appear to be nonspecific lipid binding and transfer proteins that play a minor role in the intracellular cholesterol economy (6). Recent work has identified a group of proteins termed StarD4, -5, and -6 that are structurally related to StAR and appear to play major roles in intracellular cholesterol transport (7). These proteins were discovered through their SREBP-responsive regulation and were found to have closely related cDNA, gene, and protein structures (8). One of them, StarD4, has been crystallized (9), revealing a structure essentially the same as the StAR-like domain of a protein called MLN64 (metastatic lymph node, clone 64) (10) (see below). These proteins lack sequences that target them to specific subcellular organelles; hence they seem to be cytoplasmic proteins that bind insoluble lipids, permitting the lipid to be transported across aqueous cytosol. For example, StarD5 is found in cytosol, but not in microsomes or mitochondria (11). The expression of mouse StarD4, but not StarD5, is regulated by SREBP, and both StarD4 and D5 exert low levels of StAR-like activity in COS-1 cells cotransfected with the cholesterol side-chain cleavage enzyme system (12). StarD4 is widely expressed, StarD5 is primarily expressed in reticuloendothelial cells (13), and StarD6 appears to be confined to the flagella of sperm (14). Thus the current view of steroidogenic processes is that StarD4 or a related protein is probably responsible for delivering cholesterol to the outer mitochondrial membrane (OMM) from elsewhere in the cell (lipid droplets, endoplasmic reticulum), whereas StAR itself is responsible for delivery from the OMM to the IMM.

CELLULAR REGULATION OF STEROIDOGENESIS

The net steroidogenic capacity of a cell is determined by the expression of the cholesterol side-chain cleavage enzyme, P450scc. This mitochondrial enzyme catalyzes three distinct reactions, 20α-hydroxylation, 22-hydroxylation, and scission of the 20,22 carbon-carbon bond, to convert cholesterol to pregnenolone (reviewed in Ref. 4). Each of these reactions requires donation of a separate pair of electrons from reduced nicotinamide adenine dinucleotide phosphate to P450scc via the intermediary of two electron-transfer proteins, ferredoxin reductase and ferredoxin (4). This series of reactions is slow, with a net turnover number of about six molecules of cholesterol per molecule of P450scc per second (15). Endogenously occurring (16) and genetically targeted (17) animal knockouts of P450scc confirm that this is the only enzyme that can convert cholesterol to pregnenolone. Thus it is the presence of P450scc that renders a cell steroidogenic, *i.e.* able to make steroids *de novo*, as opposed to modifying steroids produced elsewhere (*e.g.* the aromatization of androgens to estrogens by fat). Because P450scc confers the steroidogenic phenotype and is rate limiting, transcription of the CYP11A1 gene encoding P450scc is the factor that determines the net steroidogenic capacity of a cell. This transcription is regulated in both tissue-specific and hormonally responsive fashions and can be induced by both the protein kinase A and protein kinase C second messenger systems, which act on different promoter elements (18), so that long-term stimulation over the course of days will increase the content of P450scc, and the level of basal steroid produced (19), as well as

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the capacity of the cell to mount a steroidogenic response.

ACUTE REGULATION OF STEROIDOGENESIS

Unlike cells that produce polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store very little steroid. Thus a rapid steroidogenic response (*e.g*. adrenal secretion of aldosterone and cortisol in response to acute hypovolemia or the pulsing of testosterone in response to an LH surge) requires rapid synthesis of new steroid. Classic studies indicated that the induction of steroidogenesis required new protein synthesis (20–22), and early evidence indicated that the cycloheximide-sensitive step was at cholesterol flow from the OMM to IMM (23, 24). It was also proposed that a cycloheximide-sensitive factor was required for transcription of genes for steroidogenic enzymes (25, 26), but such transcription is insensitive to cycloheximide in many systems (27, 28). When an adrenal is acutely stimulated with ACTH *in vivo* or a steroidogenic cell is stimulated with cAMP *in vitro*, the first detectable event is the synthesis of a family of 37- and 30-kDa proteins seen on two-dimensional gels (29–32). Kinetic studies indicated this was one protein, sequentially undergoing phosphorylation and proteolytic cleavage (31, 32). When this protein was cloned and expressed in transfected Leydig tumor cells, it elicited a steroidogenic response similar to acute stimulation with cAMP, so that it was named the steroidogenic acute regulatory protein, StAR (33). Thus StAR is the principal cycloheximide-sensitive factor in the acute steroidogenic response.

The essential role of StAR in adrenal and gonadal steroidogenesis was established by finding recessive, loss-of-function mutations of StAR in congenital lipoid adrenal hyperplasia (lipoid CAH) (34–36). Lipoid CAH is a very severe, fairly rare disorder of steroidogenesis characterized by greatly diminished or absent synthesis of all adrenal and gonadal steroids (37). It was originally thought to be caused by a disorder in P450scc (36, 37), but this was ruled out (38), and the observation that placental steroidogenesis was unaffected in this disease (39) led to the identification of StAR mutations; thus lipoid CAH represents the StAR knockout experiment of nature (36). The distinction between the acute and chronic regulation of steroidogenesis was clarified by the two-hit model of lipoid CAH, in which some findings are due to loss of the acute StAR-mediated steroidogenic response (first hit), and others are attributable to loss of the chronic P450scc-mediated response as a consequence of cholesterol accumulation and cell death (second hit) (40). Because the ovary is steroidogenically quiescent in fetal life (41), ovarian steroidogenic cells escape the second hit until follicles are sequentially recruited after puberty, predicting that affected 46,XX females will have fairly normal pubertal development. These predictions of the two-hit model were soon confirmed by clinical observations (42, 43) and by observations in StAR-knockout mice (44, 45). Thus the study of lipoid CAH has played a central role in understanding the biology of StAR (36).

By contrast to the adrenal and gonad, the human placenta does not express StAR. Although the placenta makes large amounts of steroids, it does not mount an acute response, so there would seem to be no physiological role for an acute regulator. The capacity to make large amounts of steroids is partially explained by the large mass of the placenta, and possibly also by evidence that the placenta cleaves MLN64 (see below) to a biologically active StAR-like molecule (46).

StAR-RELATED LIPID TRANSFER (START) DOMAINS AND THE STRUCTURE OF StAR

When the sequence of StAR was initially determined (33, 47), it was thought to represent a novel, unique class of proteins. Independent studies of metastatic breast carcinoma led to the cloning of MLN64, a 445-amino acid protein, the carboxyl-terminal 227 amino acids of which were 37% identical and about 50% similar to the sequence of StAR (48). When only this StAR-like domain (N-218 MLN64) was expressed, it had about half of the ability of StAR to promote steroidogenesis, either when expressed in transfected cells or when purified and added to steroidogenic mitochondria *in vitro* (46, 49). The human placenta appears to be able to cleave a segment of MLN64 of about this size, which may stimulate placental steroidogenesis (46). Recent studies have implicated MLN64, a related protein dubbed "MENTHO," and the NPC proteins disordered in Niemann-Pick type C disease in the trafficking of cholesterol in peroxisomes and lipid droplets (50–52). The similarity in the sequences and activities of StAR and the carboxyl-half of MLN64 led to searches of computational databases, identifying similar domains in many other proteins found in a broad array of eukaryotic organisms, including plants and invertebrates (53, 54). This domain was termed the START domain, even though a role in lipid transfer has not been established for most of these.

Several groups attempted to delineate the three-dimensional structure of StAR by x-ray crystallography (unreported) and by nuclear magnetic resonance spectroscopy (55). To date, the structure of StAR has not been determined directly, but x-ray crystal structures are now available for two closely related proteins: N-216 MLN64 (10) and Star D4 (9). Computational modeling of human StAR based on these data shows a very similar structure (56, 57) (Fig. 2). The overall structure of these START domains includes two long α-helices at the N and C termini, two short

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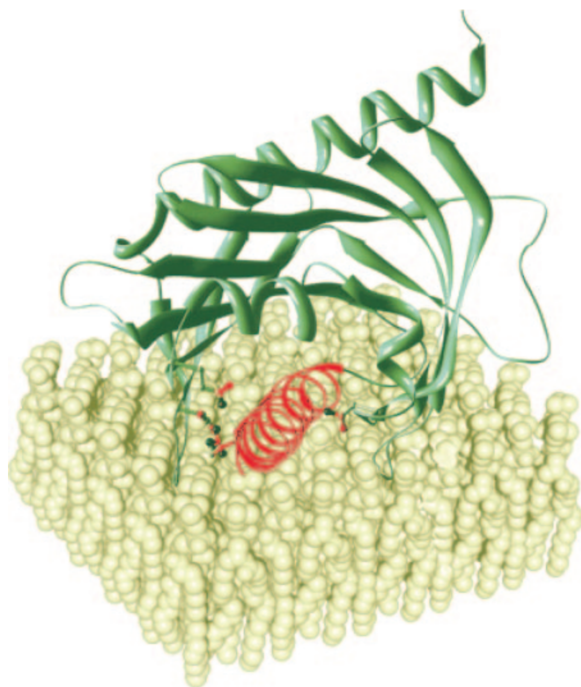

Fig. 2. Model of N-62 StAR, Shown as a *Ribbon Diagram*, Sitting on a Phospholipid Membrane Representing the OMM

The N terminus is in the *upper right-hand corner*, and the C-helix (in *red*) is in the *lower center*. Liposome protection experiments show that only the C-helix, and possibly small portions of the adjacent Ω-loops, interacts with the OMM. Arg272 in the C-helix forms a hydrogen bond with Asp106 in the Ω1-loop, each shown as *ball-and-stick figures*. This bond limits the movement of the C-helix, closing the cholesterol-binding pocket. Interaction of StAR with protonated phospholipids disrupts this (and other) hydrogen bonds, permitting the C-helix to swing to the right, opening the sterol-binding jacket. [Reproduced with permission from D.C. Yaworsky *et al*.: *J Biol Chem* 280:2045–2054 (57)].

α-helices, and a set of nine antiparallel β-sheets that form a helix-grip fold. The most notable feature is a hollow hydrophobic pocket that has appropriate dimensions and geometry to bind a single molecule of cholesterol. The pocket is defined primarily by the β-sheets and the C-helix, which forms its floor. The interior surface of the pocket contains only two charged residues; if a cholesterol molecule is modeled in the pocket, these are perfectly positioned to coordinate with the 3β-hydroxyl group of cholesterol, and direct binding assays showed that both N-218 MLN64 and N-62 StAR bind cholesterol with 1:1 stoichiometry (10).

MECHANISM OF StAR's ACTION

Multiple studies have addressed the regulation of StAR at the level of transcription and intracellular second messenger pathways (58), but progress on the key biochemical question of the mechanism of StAR's action has been modest until recently. It was clear that StAR facilitated the movement of cholesterol from the OMM to the IMM, but its mechanism of action has proven to be difficult to study. At least four models of StAR's action have been proposed: contact sites, desorption, intramembrane shuttle, and OMM molten globule.

Contact Sites

StAR is synthesized as a short-lived cytoplasmic 37-kDa protein that has a typical mitochondrial leader peptide that is cleared upon mitochondrial import to yield the long-lived intramitochondrial 30-kDa form (29–33). This logically led to the view that the 37-kDa form is a precursor and that the 30-kDa protein is the mature active form; it was proposed that mitochondrial import of the StAR protein resulted in the formation of contact sites between the OMM and IMM, permitting cholesterol from the OMM to flow down a chemical concentration gradient to the IMM, and a role for the mature 30-kDa protein in loading cholesterol onto the P450scc enzyme was considered (59). This contact site model was supported by electron microscopic evidence for increased numbers of contact sites in adrenal mitochondria stimulated with ACTH (60), and by studies with semipurified contact sites that suggest increased cholesterol trafficking at these sites (61). The role of contact sites and the role of the intramitochondrial 30-kDa protein were strongly questioned by the report that StAR remains fully active when confined to the cytoplasm. Deletional mutagenesis of StAR expression vectors showed that up to 62 amino-terminal residues of StAR, including the entire mitochondrial leader and more, could be removed without affecting StAR's activity in transfected cells (62). By contrast, deletion of only 10 carboxyl-terminal residues reduced activity by 50%, and deletion of 28 C-terminal residues ablated all activity. Immunogold electron microscopy and mitochondrial fractionation studies indicated that the N-62 StAR was confined to the cytoplasm (62). Thus it seemed that StAR had to act on the OMM.

Desorption

Studies with bacterially expressed N-62 StAR showed that this protein could transfer cholesterol from synthetic liposomes to microsomes or to mitochondria that had been pretreated with trypsin (63). This suggested that StAR alone is sufficient to transfer cholesterol to any potential acceptor membrane and that a mitochondrial acceptor protein (a StAR receptor) is not required. As a corollary it was suggested that the principal role of StAR's leader peptide is to confine its action to the mitochondrion and to keep it away from other organelles (63, 64). StAR's importation into the mitochondrion would thus terminate its action. It was proposed that StAR acts on the OMM to desorb cholesterol, knocking it out of the OMM and permitting it to traverse the mitochondrial intramembranous space

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(IMS), presumably as microdroplets, before it is taken up by the IMM (63, 65). However, no attempt to demonstrate the presence of such IMS microdroplets of cholesterol has been reported, and this model appears to make few other testable predictions. The desorption model thus posits a direct action of StAR on OMM lipids, without the intermediacy of other OMM proteins. This could be consistent with the molten globule model (below). However, because the stoichiometry of cholesterol transfer was not measured in these experiments, it was not clear whether they truly modeled events *in vivo*. More recent experiments show that the stoichiometry of transfer from liposomes *in vitro* is about two molecules of cholesterol per molecule of StAR (66), whereas the stoichiometry of transfer *in vivo* is approximately 400:1 (67). Thus the desorption model does not appear to account for StAR's action.

IMS Shuttle

The determination of the structure of N-216 MLN64 (and by inference StAR), which identified a cholesterol-binding pocket, and the demonstration that StAR could bind cholesterol led to the conclusion that StAR must reside in and act in the mitochondrial intramembranous space (IMS), where it would shuttle cholesterol one molecule at a time from the OMM to the IMM (10). Because this proposal was inconsistent with the observations that N-62 StAR is active on the OMM (62, 68), those data were dismissed as an artifact of *in vitro* overexpression, and because the structure indicates StAR was a cholesterol-binding protein, the proposal that StAR requires a molten globule conformational change was dismissed as illogical (10). Despite a lack of experimental evidence for this IMS shuttle model, it was initially accepted by many investigators because of its logic, simplicity, and structural basis.

Molten Globule

Biophysical studies of purified, bacterially expressed human N-62 StAR and several StAR mutants that cause lipid CAH suggested that subtle changes in the folding of the StAR protein could have substantial effects on StAR activity (69). Further studies based on circular dichroism (CD) and fluorescence spectroscopy and partial proteolysis characterized by mass spectrometry, indicated that the carboxyl-half of StAR, the region containing almost all disease-causing missense mutations, was loosely folded and protease-sensitive, whereas the amino-terminal half was tightly folded and protease resistant (70). This suggested that the carboxy-terminal half of StAR was especially important for its function. Spectroscopic studies showed that StAR underwent a conformational change at approximately pH 3.5 that was characterized as a "molten globule transition" (70). Molten globules are partially unfolded proteins that have lost some tertiary structure but retain their secondary structure (array of α-helices and β-sheets) (71). When a protein is denatured (unfolded), it becomes so in steps initially characterized by loss of some, but not all, of its tertiary structure. Many membrane-associated proteins undergo molten-globule transitions when they exit the aqueous cytoplasm and become inserted into lipid membranes by undergoing conformational changes that bring hydrophobic residues to the surface to interact with membrane lipids. Laboratory evidence for a molten globule state of a protein can include changes in the spectroscopic behavior (especially changes in the CD spectra) and increases in sensitivity to partial proteolysis. Whereas pH 3.5 is not achieved within a cell, pH 3.5 in a cuvette might model events when StAR interacts with the protonated phospholipid head groups of the OMM. Thus the molten globule model proposes that StAR acts exclusively on the OMM and that its activity requires a change in protein conformation elicited by protonated phospholipids, as modeled by pH 3.5 *in vitro* (70). Support for this model comes from observations that an intact mitochondrial proton pump is required for StAR activity (72–74) and from the observation that the molten globule transition at pH 3.5–4.0 was also seen in association with phospholipid membranes that recapitulate the composition of the OMM (75).

StAR ACTS EXCLUSIVELY ON THE OMM

To determine StAR's site of action and hence discriminate among the models outlined above, constructs were designed to affix StAR on the cytoplasmic side of the OMM, in the IMS, or on the matrix side of the IMM, where P450scc resides (Fig. 3). This was achieved by fusing N-62 StAR to various mitochondrial proteins that have well-defined topologies (76). To immobilize StAR on the cytoplasmic side of the OMM, N-62 StAR was fused to the carboxyl terminus of a protein called Tom20, which has 50 N-terminal residues buried in the OMM and 95 residues exposed to the cytoplasm (77). Cells expressing the Tom20/StAR fusion achieved maximal constitutive steroidogenesis (76). A similar strategy, using an IMS protein termed Tim9 (78), showed that StAR was inactive in the IMS. When this Tim9/StAR fusion protein was prepared and folded *in vitro*, and then added to steroidogenic mitochondria, it was active, demonstrating that it was the IMS location of the Tim9/StAR fusion, not its structure, that rendered it inactive in transfected cells (76). Similarly, when StAR was localized to the mitochondrial matrix, it was also inactive (76).

Manipulations of the StAR leader peptide in conjunction with mitochondrial protein-import assays permitted an independent test of the view that StAR acts exclusively on the OMM (Fig. 3). Constructs that slowed StAR's mitochondrial entry had increased activity and those that sped up its entry had decreased activity, indicating that the level of StAR activity was determined by its OMM occupancy time (76). Finally, it

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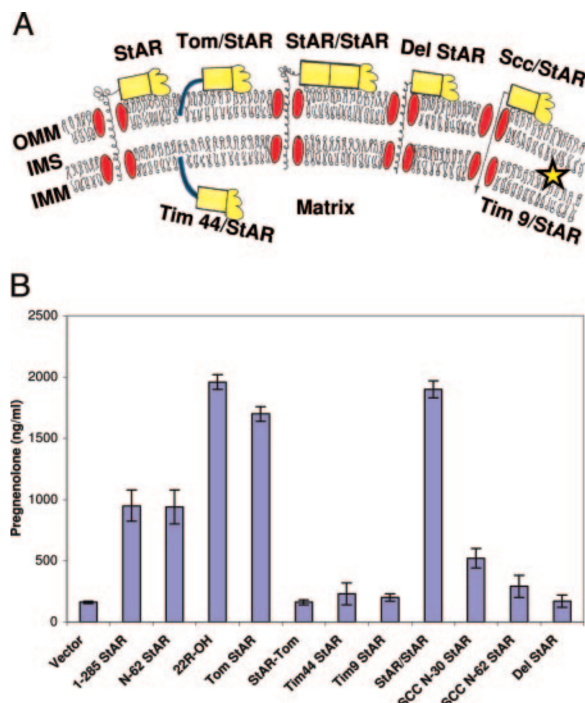

Fig. 3. StAR Acts on the OMM

A, Experimental design, depicting the OMM, IMS, and IMM. The initial 62 residues of StAR are represented as the *corkscrew line*, the protease-resistant domain (residues 63–188) as the *yellow rectangle*, and the protease-sensitive carboxyl-terminal domain as the *yellow shape to the right of the rectangle*. The mitochondrial protein-import machinery is represented by the *red ovals*. Tom/StAR comprises N-62 StAR fused to the C terminus of Tom20, thus immobilizing StAR on the OMM; Tim9/StAR similarly immobilizes StAR in the IMS and Tim44/StAR immobilizes StAR on the matrix side of the IMM. The StAR/StAR construct fuses 1–188 StAR to N-62 (63–285) StAR, thus dimerizing the protease-resistant domain; Del (deleted) StAR retains residues 1–30 but deletes a pause sequence at residues 31–62, and Scc/StAR replaces the StAR leader (either 1–30 or 1–62) with the 39-amino acid leader sequence of human P450scc (© W. L. Miller). B, Activity of the constructs depicted in panel A, when transfected into COS-1 cells cotransfected with the F2 fusion of the cholesterol side-chain cleavage system (101). A low level of StAR-independent steroidogenesis is seen with the vector control, but full-length (1–285) or N-62 StAR increase steroidogenesis 6-fold. The hydroxysterol 22R-OH-cholesterol bypasses the action of StAR (102), providing an index of the maximal level of steroidogenesis that can be achieved in this system. Transfection with the Tom/StAR fusion or the StAR/StAR dimmer (panel A) achieves this maximal level of steroidogenesis; by contrast, when StAR is placed at the N terminus rather than the C terminus of Tom20 (StAR/Tom), when it is localized to the matrix side of the IMM (Tim44/StAR), or when it is confined to the IMS (Tim9/StAR), it is inactive. Replacing StAR's leader peptide with that of P450scc also reduces activity; cell-free transcription/translation linked to mitochondrial import assays show that the level of activity seen with the leader mutants is inversely proportional to their speed of import.

had been suggested that the activity of N-62 StAR in transfected cells was an artifact of cellular overexpression (10). However, immunoquantitation of the StAR produced in transfected cells established that the studies of N-62 StAR achieved physiological, not pharmacological, levels of expression (76). These studies established that StAR acts exclusively on the OMM, thus excluding the IMS/shuttle model of StAR's action.

ONLY THE C-HELIX OF StAR INTERACTS WITH THE OMM

The proof that StAR acts exclusively on the OMM limited models of its action and raised additional questions. Understanding the interaction of StAR with the OMM required understanding of what parts of the StAR molecule associate with the OMM and which residues are involved, so that one could visualize the geometry of this interaction. Small unilamellar vesicles (SUVs), a type of liposome, were prepared composed of the same array of lipids and cholesterol as an OMM, and mixed with pure, bacterially expressed, biologically active human N-62 StAR (57). The StAR/SUV mixture was then subjected to proteolysis, and the peptides were recovered and analyzed by mass spectrometry. Comparing the peptides obtained in the presence or absence of SUV and under different pH conditions permitted identification of the residues of StAR that are associated with the lipid membrane, thus inhibiting proteolysis. Coupling these data with a computational model of human StAR based on the crystallographic structure of N-216 MLN64 permitted visualization of StAR/OMM interaction. These liposome protection experiments show that only the outside aspect of the carboxyl-terminal helix (C-helix) is reproducibly associated with the membrane (Fig. 2). Not surprisingly, this helix is amphipathic; most of its residues are charged and interact with the OMM, but its hydrophobic F267, L271, and L275 residues are oriented toward the cholesterol-binding pocket (Fig. 4A). Proteolysis of N-62 StAR and of a synthetic peptide model of the C-helix confirm that this association is more avid at pH 3.5–4.0 than it is at pH 7, providing independent evidence for the pH-dependent molten globule model (57). Thus these data indicate that StAR does not become deeply buried in the OMM, as one might envision as necessary to pick up a cholesterol molecule, but instead treads lightly on the OMM.

VISUALIZING THE MOLTEN GLOBULE BY MOLECULAR DYNAMICS (MD)

The crystal structure of N-216 MLN64 shows that, although the cholesterol-binding pocket can accommodate one cholesterol molecule, there was insufficient room for a cholesterol molecule to enter or exit, mandating a conformational change associated with cholesterol uptake. The liposome protection data showed that the C-helix forms the floor of the choles-

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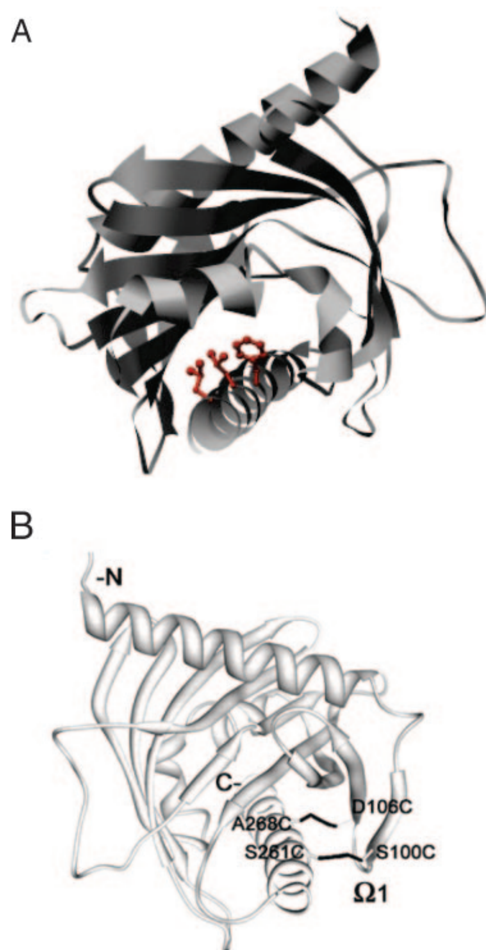

Fig. 4. Applications of the Model of StAR

A, Model from the same perspective as Fig. 2, showing the orientation of the hydrophobic F267, L271, and L275 residues toward the sterol-binding pocket. B, Model of N-62 StAR showing the locations of the SS mutant replacing Ser100 and Ser261 and the DA mutant replacing Arg106 and Ala268 with Cys. Both mutants are shown in a single image, but each was prepared separately. Note that the perspective is from behind the molecule, as compared with Figs. 2 and 4A. [Panel B is reproduced with permission from B. Y. Baker *et al*.: *J Biol Chem* 280:41753–41760 (79)].

terol-binding pocket and is the region of the protein that is most likely to move during StAR action. The use of computational MD simulations permit one to visualize how StAR's conformation might change in association with the OMM and at acidic pH, *i.e*. to visualize the molten globule model (79). A crystal structure is rigid and fixed in space, analogous to conceptualizing the protein at absolute zero, where nothing moves. MD is a computational approach that permits one to visualize protein movement in real time under physiological conditions. After optimizing a computational model of a protein by energy minimization, the model is heated to 300° K by permitting the atoms in the model to acquire vibrational energy. The location of each atom in the protein and the angle, distance, and energy of each bond are calculated every 2 fsec for a total of 3 nsec. The calculations include all the atoms in the protein plus all the atoms in water box, a computational box of water molecules designed to solvate the protein so that no atom in the protein comes closer than 10 Å to the edge of the box. For N-62 StAR this included 3,375 protein atoms and 28,893 water atoms; each 3-nsec run takes about 1 month, running in the background on a mainframe computer.

MD simulations were done for N-62 StAR under three conditions (79). First, the default settings for the chemistry of each amino acid were used, corresponding to approximately pH 7; second, all the Asp, Glu, and His residues were protonated to model the conditions achieved at pH 3.5 in solution; third, only those Asp, Glu, and His residues shown by the liposome protection experiments to be in contact with the SUV were protonated, thus modeling the interaction of StAR with the protonated phospholipid head groups of the OMM. When contemplating the effects of pH on individual protein molecules, one must remember that the concept of pH applies only to large numbers of molecules: at pH 7, a box measuring 100Å on each edge should contain 6×10^{-5} protons! Hence the relevant question is not pH, but whether or not a carboxylic acid side group is protonated. Whereas the data modeling pH 7 show that the structure remains fairly stable, the data at pH 3.5 show that the C-helix moves substantial distances, opening and closing the sterol-binding pocket. Essentially the same results are seen when the entire protein is acidified, which models the conditions used for the experiments that led to the molten globule model, and when only the C-helix is acidified, modeling the interaction of StAR with the OMM (79). These data show that protonation of the C-helix is sufficient to elicit the molten globule transition.

DIRECT CONFIRMATION OF THE MOLTEN GLOBULE

To test whether this computationally seen movement of the C-helix is required for StAR activity, we designed mutants that would immobilize the C-helix, preventing the movement that opens the cholesterol-binding pocket. Molecular modeling indicated that when two residues, Ser100 and Ser261, are changed to Cys, the two Cys residues would form a disulfide bond that would reduce the movement of the C-helix; similarly, the modeling predicted that changing Asp106 and Ala268 to Cys would create a disulfide bond that would eliminate almost all movement of the C-helix. These S100C/S261C (SS) and D106C/A268C (DA) mutants were chosen from many possible disulfide mutants because computational analysis indicated that the SS and DA mutants would not disrupt other aspects of the protein's structure (79). The SS mutant has a disulfide midway along the C-helix and is

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predicted to permit some residual movement of this helix, whereas the DA mutant is near the C terminus of the C-helix and is predicted to prohibit all movement of the helix (Fig. 4B). These mutants were built, expressed in bacteria, purified to homogeneity, and assessed by mass spectrometry to demonstrate that the disulfides were in the correct positions (79). Analysis of the mutants by CD spectroscopy under various pH conditions showed that they had lost most of the features that characterize the pH-dependent molten globule transition of wild-type StAR; thus these mutants had the same size, shape, and geometry as StAR but should be less flexible in an acidic environment.

As predicted by the modeling, the SS mutant retained partial capacity to bind cholesterol, and the DA mutant was devoid of cholesterol-binding capacity. Similarly, when the recombinant proteins were tested with steroidogenic mitochondria *in vitro*, the SS mutant retained partial activity but the DA mutant had no activity. Finally, full cholesterol-binding and steroidogenic activities could be restored by disrupting the disulfide bonds with dithiothreitol, indicating that it is the restricted movement of the C-helix, and not the amino acid replacements, that is responsible for the decreased activity of these mutants (79) (Fig. 5). Thus StAR acts by interacting with the OMM where protonated phospholipids protonate residues that form hydrogen bonds between the C-helix and the Ω1 loop, permitting the helix to swing to the side opening the cholesterol-binding pocket. These experiments provide powerful evidence in support of the original molten globule model of StAR's action (70). Thus all presently available data are consistent with StAR acting exclusively on the OMM and requiring an acidification-induced conformational change to act.

PARTNERS IN StAR's ACTION

The demonstration that StAR acts exclusively on the OMM (76) raises difficult questions. First, StAR appears to act as a cholesterol-binding protein on the OMM, and bacterially expressed N-62 StAR can bind and transfer cholesterol between synthetic liposomes *in vitro* (66). However, *in vitro* StAR transfers cholesterol with only 2:1 stoichiometry (66), but *in vivo* each molecule of StAR fosters the delivery of up to 400 molecules of cholesterol to the IMM (67). It is not clear how each StAR molecule moves hundreds of molecules of cholesterol from the OMM to the IMM, while the StAR remains on the OMM. Second, it is difficult to conceptualize how StAR, interacting loosely with the OMM, induces the movement of cholesterol across two membranes. The role of StAR as a cholesterol-binding protein is strongly supported by its cholesterol binding capacity (10) and by the close correlation between cholesterol binding and activity (79). Understanding how StAR acts requires knowing at least two things that remain unclear: where does the cholesterol come from and what other proteins or other factors contribute to the mitochondrial import of cholesterol. Early studies of kinetic pools of mitochondrial cholesterol suggested that there is a stable pool of OMM cholesterol (presumably the cholesterol that is associated with the membrane and required for its fluidity), and a labile pool of cholesterol which, although chemically indistinguishable from stable cholesterol, is nevertheless physically and kinetically more accessible (80). Domains of plasma membranes rich in cholesterol (rafts) are well described; whereas discussions of rafts usually concern the plasma membrane, newer concepts suggest that rafts may be found in any cellular membrane (81). It appears likely that cholesterol is not homogeneously distributed in membranes such as the OMM but associates into lipid-rich domains (82). Some evidence indicates that StAR preferentially associates with ordered membranes that contain cholesterol (75); hence OMM rafts may be relevant to steroidogenesis. Another possibility is that the labile pool of cholesterol is specifically associated with another protein. Numerous searches for StAR-interacting proteins have been attempted using technologies

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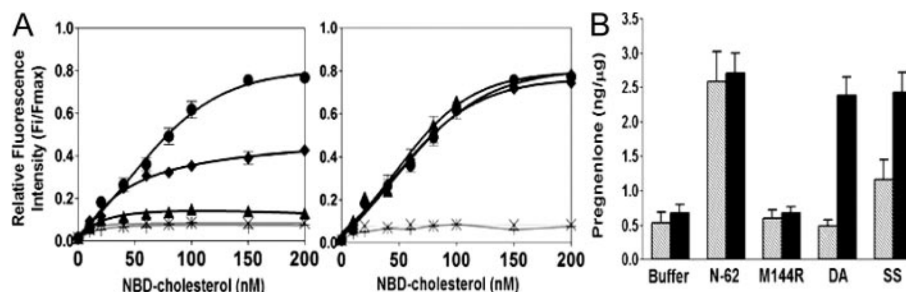

Fig. 5. Activity of the Disulfide Mutants

A, Binding of fluorescent NBD-cholesterol by wild-type (●), SS (♦), and DA N-62 StAR (▲), under native conditions (*left*) and after reduction with dithiothreitol (*right*). Data for the inactive StAR mutant M144R (x) and for the buffer without protein (+) are shown in *gray*. B, Capacity of the wild-type and SS mutant and DA mutant of N-62 StAR to induce steroidogenesis, as assessed by the capacity of each protein to promote steroidogenesis in mitochondria isolated from mouse Leydig MA-10 cells. *Gray bars* represent native protein; *black bars* indicate protein reduced with dithiothreitol. [Reproduced with permission from B. Y. Baker *et al*.: *J Biol Chem* 280:41753–41760 (79)].

such as coimmunoprecipitation, glutathione-*S*-transferase pulldowns, and yeast two-hybrid screening. A report of the cloning of a StAR-binding protein identified by a yeast two-hybrid approach (83) may be an artifact, because the cloned protein is a factor involved in psoriasis (84, 85).

However, pharmacological evidence has suggested that another protein, the peripheral benzodiazepine receptor (PBR), also plays a key role in mitochondrial import of steroidogenic cholesterol (86). Although PBR is expressed ubiquitously, it is most abundant in steroidogenic cells (87); agents that bind to PBR stimulate steroidogenesis and induce cholesterol movement from the OMM to the IMM (88, 89), and targeted disruption of PBR in rat Leydig R2C cells reduced steroidogenesis (90), but PBR-knockout mice are embryonic lethal (91). PBR is a small, 18-kDa OMM protein with five-transmembrane domains (92), having a cytoplasmically exposed carboxyl terminus containing a cholesterol recognition amino acid consensus (CRAC) domain. Mutagenesis of this domain disrupts cholesterol binding and transfer to the IMM (93, 94). It is attractive to consider that StAR and PBR interact. One study using fluorescence resonance energy transfer indicated that StAR and PBR approach within 100Å of each other during StAR-induced steroidogenesis (95); however, these experiments did not establish protein-protein contact and do not exclude the possibility that one or more additional proteins are involved in this putative interaction.

Although a physical interaction between StAR and PBR has not been established, powerful evidence for a functional interaction between StAR and PBR comes from recent experiments using the Tom20/StAR fusion protein (96). Mitochondria from mouse Leydig MA-10 cells that express the Tom20/StAR fusion produce steroids at a maximal level (76), but if the cells are treated with oligonucleotides antisense to PBR, their steroidogenic capacity is lost; returning functional PBR to these mitochondria *in vitro* using cell-free transcription/translation of PBR restored their steroidogenic capacity (96). Furthermore, restoring N-terminally deleted PBR, which retains the CRAC domain but lacks the drug-ligand domain, was nearly as effective as full-length PBR, but PBR carrying the Y153S mutation that disrupts cholesterol binding to the CRAC domain was inactive (96). Thus these experiments demonstrate a functional interaction between StAR and PBR and a role of the CRAC domain in steroidogenesis. The predominant view is that StAR delivers cholesterol to PBR (91). However, this view appears to be inconsistent with experiments in which bacterially expressed StAR stimulates steroidogenesis from isolated mitochondria *in vitro* (46, 68, 76); it appears more likely that the CRAC domain of PBR contains the reservoir of labile cholesterol available for steroidogenesis and that StAR acts to mobilize this pool. It is also not clear whether other proteins participate in this action. Studies of the action of PBR in response to benzodiazepines indicate that PBR associates with two other proteins, the voltage-dependent anion channel (VDAC) and the adenine nucleotide-binding protein (97). Several studies implicate VDAC in cholesterol import, and VDAC is found at contact sites between the OMM and IMM (98), but a role of adenine nucleotide-binding protein is less clear (99, 100).

CONCLUSIONS

Although N-62 StAR can deliver cholesterol to a wide variety of cellular membranes, StAR is not the principal agent delivering cholesterol to the OMM; that role is probably played by StarD4. StAR's principal action is to move cholesterol from the OMM to the IMM. Recent data show that StAR achieves this action on the OMM, where it must undergo a conformational change that probably results from its interaction with protonated OMM phospholipids. Although StAR is essential, it does not act alone; several lines of evidence indicate that its action requires PBR. The nature of the interaction between StAR and PBR is not established, and it is not clear whether VDAC or other proteins participate in the cholesterol-import machinery.

Note Added In Proof

Since the acceptance of this manuscript in September 2006, two additional studies have been published that concern the mechanism of StAR's action. First, Liu *et al*. (103) have used a photo-affinity cross-linking tactic to identify a macromolecular complex consisting of StAR, PBR, and two other proteins, PAP7 (PBR-associated protein 7) and PKARIα (protein kinase A regulatory subunit Iα), providing compelling evidence for a direct interaction between StAR and PBR on the OMM. Second, Murcia *et al*. (104) have reported MD simulations of the docking of cholesterol with StAR. These simulations confirm the observation that movement of the C-helix and its adjacent loops is required for cholesterol to enter or exit the SBP (79) but differ slightly in emphasizing the movement of the loop between residues 172 and 182 rather than the loop between residues 102 and 106. These differences probably reflect differences in the computational programs employed, the use of "implicit solvent" and MD confined to default (pH 7) conditions (104).

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