Steroidogenic Acute Regulatory Protein-binding Protein Cloned by a Yeast Two-hybrid System*

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Steroidogenic acute regulatory (STAR) protein plays a key role in the transport of cholesterol from the outer mitochondrial membrane to the inner membrane. A STAR mutant protein lacking the first 62 amino acids (N-62 StAR protein) has been reported to be as effective as wild-type StAR protein. In the present study, we examined the mechanism by which STAR protein stimulates steroidogenesis. A GAL4-based yeast two-hybrid system was used to identify proteins interacting with N-62 STAR protein. Nine positive clones were obtained from screening 1 × 10⁶ clones. The results of pull-down assays and mammalian two-hybrid assays confirmed interaction between N-62 STAR protein and the clone 4 translated product. The clone 4 translated product was named STAR-binding protein (SBP). We prepared an expression plasmid (pSBP) by inserting SBP cDNA into the pTarget vector. After cotransfection with the human cytochrome P450scc system, STAR expression vector, and pSBP, the amount of pregnenolone produced by COS-1 cells was increased. The amount of steroid hormones produced by steroidogenic cells subjected to small interfering RNA treatment was less than that produced by control cells. In conclusion, SBP binds StAR protein in cells and enhances the ability of StAR protein to promote syntheses of steroid hormones.

The first step in the biosynthesis of steroid hormones is the conversion of cholesterol into pregnenolone. The rate-limiting process is the transport of cholesterol from the outer mitochondrial membrane to the inner membrane, where cytochrome P450 side-chain cleavage (P450scc) enzyme resides. Steroidogenic acute regulatory (STAR) protein plays a key role in the intra-mitochondrial movement of cholesterol (1). Mutations in the StAR gene cause congenital lipoid adrenal hyperplasia, a condition in which cholesterol accumulates in cytoplasmic lipid droplets, and adrenal and gonadal steroidogenesis is severely impaired (2, 3). StAR gene knockout mice have the same phenotype as that of humans with congenital lipoid adrenal hyperplasia, that is cholesterol accumulation predominantly in the adrenal gland and markedly reduced steroid hormone secretion (4, 5).

The tropic hormones ACTH, luteinizing hormone, and follicle-stimulating hormone stimulate steroid hormone production in the adrenal glands and gonads through a cAMP-dependent pathway. StAR gene expression increased rapidly in response to cAMP stimulation (6, 7). Although the human StAR gene promoter lacks cAMP-responsive elements, steroidogenic factor-1 (8), CCAAT/enhancer-binding proteins, and GATA-4 confer cAMP-dependent StAR gene expression (9–11). The factors SREBPs, Sp1, insulin-like growth factors, transforming growth factor-β, tumor necrosis factor-α, DAX-1, and SIK have also been shown to regulate StAR gene expressions (12–19). Increased progesterone synthesis prior to an increase in the level of StAR mRNA expression in response to cAMP induction has been revealed by analysis of hyperacetylation in the StAR gene promoter (20). Although this increased steroidogenesis is thought to be the result of phosphorylation of the StAR protein by protein kinase A (8), the mechanism is still not clear.

Following synthesis, the 37-kDa StAR pre-protein is imported into mitochondria with subsequent cleavage of the mitochondrial targeting sequence, yielding a 30-kDa mature StAR protein (21, 22). StAR mutant protein lacking the first 62 amino acids (N-62 StAR protein), which contain the mitochondrial targeting sequence, has been reported to be as effective as wild-type StAR protein in stimulating steroidogenesis (23). This led to the conclusion that the C terminus of StAR protein encodes its biological function for steroidogenesis and that the StAR protein acts on the outer mitochondrial membrane to stimulate cholesterol translocation. The proposed site of action of StAR protein on the cytoplasmic face of the outer mitochondrial membrane raised questions about the mechanisms by which StAR protein trades with mitochondria and expresses its action in the cytoplasm. In the present study, we used a yeast-two hybrid system to screen for proteins that interact with StAR protein and modulate its steroidogenic action.

MATERIALS AND METHODS

Plasmid Constructs—A plasmid expressing a GAL4-N-62-StAR fusion lacking 62 amino acid-terminal residues was constructed by inserting an EcoRI fragment, prepared by PCR using human StAR cDNA as a template, into a pACT2 vector, which has a GAL4-activating domain (GAD) (Clontech Laboratories, Inc., Palo Alto, CA). Plasmids expressing GAL4-stAR mutants (GAL4-R193X, GAL4-Q253X, and GAL4-frame shift) were also constructed by inserting an EcoRI fragment prepared by PCR from cDNA of human StAR mutants, which were previously constructed (2). A plasmid expressing a GAL4-N-62-StAR fusion was constructed (2). A plasmid expressing a GAL4-N-62-StAR fusion was...
also constructed by inserting an EcoRI fragment prepared by PCR and cloned into a pACT2 vector, which has a GAD (Clontech). Plasmid pVP16-StAR was constructed by inserting the EcoRI fragment from human N-62 Star cDNA into the pVP16 vector, which has an activation domain (Ad) derived from the VP16 protein of herpes simplex virus. The 4 clone translated product was named StAR-binding protein (SBP). We produced plasmid pM-SBP by inserting the EcoRI/BamHI fragment from SBP cDNA into the pM vector, which has an Ad GAL4 DNA-binding domain (DNA-BD). Reverse combinations were also prepared; pVP16-SBP was constructed by inserting the EcoRI/BamHI fragment of SBP into the pVP16 vector. To produce plasmid pM-StAR, the EcoRI fragment from N-62 Star was cloned into the pM vector. pG5lac (Promega Corp., Madison, WI) contains the chloramphenicol acetyltransferase gene or the luciferase gene as a reporter. From RACE results, we prepared an expression plasmid (pSBP) by inserting the EcoRI fragment of the entire coding region amplified by PCR from testis cDNA into the pTarget vector (Promega). A plasmid expressing an SBP-GFP fusion protein was constructed with the N-terminal green fluorescent protein (GFP) (pSBP-GFP) by inserting an EcoRI fragment prepared by PCR from the full-length cDNA of human StAR cDNA into pDsRed2-N1 (Clontech). The plasmids were prepared for transfection studies using a Qiagen Maxiprep system (Qiagen, Hilden, Germany).

Cell Culture—Mouse Y1 adrenal tumor cells, COS-1 cells, and human Hep G2 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). Human adrenocortical carcinoma H295R cells and mouse MA-10 Leydig cells were a gift from Dr. Mitsuhiro Okamoto, Osaka University Medical School (Osaka, Japan). Human MCF-7 breast cancer cells were obtained from ATCC (Manassas, VA). Human granulosa-like tumor KGN cells were a gift from Dr. Yoshihiro Nishi, Graduate School of Medical Sciences, Kyushu University (24). The Y-1 cells and COS-1 cells were grown in 35-mm plastic dishes. The Y-1 cells and COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 50 mg/ml gentamicin. The Y-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 50 mg/ml gentamicin. H295R cells were grown in DMEM/F-12 containing 2% ULMOTERO G (BioSepra, Cergy-Pontoise, France) and 1% ITS Premix (BD Biosciences).

Yeast Two-hybrid Interaction Screening—A human testis cDNA library (Clontech) in the activation domain vector pACT2 was amplified using the recommended protocol of the manufacturer (Clontech). To identify SBP clones, the human testis cDNA library in pACT2 was introduced into the yeast reporter strain CG-1945 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-1, leu2-3, 112, gal4-542, gal80-538, cyh2, LYS2::GAL1-lacZ, HIS3, URA3::gal7-lact, ARS1::GAL1-lacZ, TRP1, TRP1::GAL1-lacZ, MATCENNA2::GAL1-lacZ) (MATCHMAKER Two-Hybrid System 2, Clontech) bearing a plasmid expressing His-tagged Star cDNA (GAL4-StAR protein) and selected on 1% adenine plates to generate a pool of 106 transformants. pM-StAR was amplified as described previously (25). The plasmid pVP16-StAR fusion proteins were cloned into the pTarget vector (Promega). A plasmid expressing an SBP-GFP fusion protein was constructed with the N-terminal green fluorescent protein (GFP) (pSBP-GFP) by inserting an EcoRI fragment prepared by PCR from the full-length cDNA of human StAR cDNA into pDsRed2-N1 (Clontech). The plasmids were prepared for transfection studies using a Qiagen Maxiprep system (Qiagen, Hilden, Germany).

RT-PCR Analysis—Total RNA was isolated from Hep G2 cells, KGN cells, H295R cells, and MCF-7 cells. Complementary DNA synthesis was carried out at 37 °C for 60 min using 150 pmol of oligo(dT) as a primer, 1 μg of total RNA, and 200 units of SUPERSCRIPT II RNase H (Invitrogen). Reverse transcriptase in a 20-μl reaction mixture contained 50 nmol of dNTPs, 20 units of SuperScript II, 10 μg of total RNA, and 150 pmol of each primer. The reaction mixtures were incubated for 1 h at 37 °C and then held at 4 °C. The reaction products were sequenced, and the sequences were compared with the human genome sequence.

Transfection and Pregnenolone Immunoassay—CO2 cells were transfected with F2, a vector for the cytochrome P450 cholesterol side-chain cleavage system (called the F2 system) kindly provided by Dr. Walter L. Miller of the University of California, San Francisco (26), pStAR (pSPORT STAR cDNA) (2), and pSBP using FuGENE 6. The cells were incubated for 48 h following transfection. Some dishes were treated with 22(R)-hydroxysterol (1 μM) during the final 24 h of culture. Forty-eight hours after transfection, the medium was collected for radioimmunoassay of pregnenolone. The assay results were normalized by serum pregnenolone concentrations produced by cultures with no additions. The results are expressed as pg of pregnenolone per mg of protein 1 h.
A GAL-4 based yeast two-hybrid system was used to identify the protein that interacts with StAR protein. A vector expressing a GAL4-StAR fusion protein containing human N-62 StAR protein, which lacks 62 amino acid terminal residues, was used to screen a human testis cDNA library in yeast strain CG1945. Nine positive clones were obtained from screening 1 × 10^6 clones. HCR, α-helix coiled-coil rod homologue; RAB5EP, rabaptin-5; NUCB2, nucleobindin 2.

| Clone          | Insert size | Identity | Colony color |
|----------------|-------------|----------|--------------|
| 1              | 2.3         | HCR      | Positive     |
| 5              | 2.2         | HCR      | Positive     |
| 11             | 3.8         | RAB5EP   | Negative     |
| 26             | 2.6         | NUCB2    | Positive     |
| 36             | 0.8         | HCR      | Negative     |
| 44             |             |          |              |
| 45             | 4.3         | RAB5EP   | Negative     |
| 49             | 2           | HCR      | Positive     |

5′-GGC GCC CGU UGU AGGA UUCG GdT dT3′ and 5′-GCA UCC CAA AGG GCG CdT dT3′. The oligonucleotides were annealed according to the Dharmacon protocol. Three hundred pmol of each duplex was introduced into cells using 15 μl of metalfectene (Biontex Laboratories GmbH, Munich, Germany) as recommended by the manufacturer. Dishes for H295R cells were treated with 20 μl tristolane (provided by Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) 6 h after transfection to inhibit the enzyme activity of 3β-hydroxysteroid dehydrogenase, which transforms pregnenolone into progesterone (27). 48 h after transfection, cells were collected for radioimmunoassay of steroid hormones. Before transfection, cells were seeded on coverslips in culture dishes. Cells were transfected using FuGENE 6 according to the manufacturer’s instructions. After transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, each coverslip was mounted onto a glass microscope slide for image capture. Adobe Photoshop 5.0 (Adobe System Inc., San Jose, CA) was used for image processing.

RESULTS

Yeast Two-hybrid Screening of Proteins Interacting with StAR Protein—A Gal4-based yeast two-hybrid system was used to identify proteins interacting with StAR protein. A plasmid expressing a GAL4-StAR fusion protein, containing human N-62 StAR protein lacking 62 amino acid-terminal residues, was used to screen a human testis cDNA library in yeast strain CG-1945. Nine clones were obtained from screening 1 × 10^6 clones. DNA sequence and data base analysis revealed that these clones could be divided into three groups: α-helix coiled-coil rod homologue (HCR, GenBank accession number NM 019052), rabaptin-5 (RAB5EP, accession number NM 004703), and nucleobindin 2 (NUCB2, accession number NM 005013) (Table I). Both HCR and NUCB2 expressed the LacZ phenotype. From the expected intracellular localization of proteins encoded in the clones, we chose clone 4, which contained a 2.3-kb insert encoding a putative cytoplasmic protein, for analysis. Clone 4 contained an open reading frame of 1971 nucleotides encoding a 657-amino acid protein and a 62-nucleotide 3′-untranslated sequence that ended in a poly(A) tail preceded 21 nucleotides upstream by an AATAAA sequence. Other clones were not examined in this study.

Interactions of StAR Protein and Clone 4 in the Yeast Two-hybrid Assay—To determine the interaction between clone 4 and StAR protein in vivo, a plasmid expressing a GAD-clone 4 fusion and a plasmid expressing GAL4-N-62-StAR and GAL4-StAR mutant fusions were examined. Transfections were also performed with the reverse combinations of clone 4 fused to GAL4 and N-62 StAR fused to GAD. The yeast transfected with StAR, and clone 4 hybrid vector expressed the LacZ phenotype, but the yeast transfected with a StAR mutant and clone 4 did not express the LacZ phenotype (Table II), confirming that N-62 StAR interacts with clone 4 in the yeast two-hybrid system.

Direct Interaction between StAR Protein and Clone 4 in Vitro—Pull-down assays were performed to examine the direct interaction between clone 4 and StAR protein. In vitro translated clone 4 (50 μl) was incubated with His-tagged CBD Cloning the yeast transfected with StAR and clone 4 hybrid vector expressed the LacZ phenotype, but the yeast transfected with a StAR mutant and clone 4 did not express the LacZ phenotype (Table II), confirming that N-62 StAR interacts with clone 4 in the yeast two-hybrid system.

Interaction between SBP and StAR Protein in Cells—To confirm further interaction between SBP and StAR protein, we used two-hybrid assays in Y-1 cells, COS-1 cells, and MA-10 cells. The empty vectors, GAL4 DNA-BD (pM) and AD (pVP16), did not activate the reporter genes. Cotransfection of GAL4-DNA-BD (pM) and AD-StAR (pVP16-StAR), pM and AD-SBP (pVP16-SBP), GAL4 DNA-BD-SBP (pM-SBP) and AD (pVP16), and GAL4 DNA-BD-StAR (pM-StAR) and AD (pVP16) did not increase reporter activity. However, the GAL4 DNA-BD-StAR (pM-StAR) and AD-SBP (pVP16-SBP) fusion proteins induced 100-fold greater activation of the promoter compared with the activity observed when the pM and pVP16 vectors were co-transfected into Y-1 cells. The switched domain constructs, pM-SBP and pVP16-StAR, also activated the reporter in Y-1 cells (Fig. 2A). Cotransfection with pM-StAR and pVP16-SBP increased the promoter activity by 100-fold in COS-1 cells (Fig. 2B).
SBP mRNA Expression in Human Tissues—Northern blot analysis was performed to examine the expression of SBP. Northern blots that each contained 2 μg of poly(A)$^+$ RNA isolated from the indicated tissues were probed sequentially with SBP and β-actin cDNAs. Expression of the SBP gene was detected in all tissues examined. Its expression level was particularly high in the testis, where the sizes of transcripts were 2.4 and 3.8 kb (Fig. 3). To examine the expression of SBP in various cell lines, we performed RT-PCR using mRNA extracted from Hep G2 cells, KGN cells, H295R cells, and MCF-7 cells (human breast cancer cells). The sizes of PCR products were 400 bp of SBP.

Identification of the Transcription Start Sites of SBP Gene Using Rapid Amplification of cDNA Ends (RACE)—For determination of the transcription start sites of the SBP gene, we employed a method based on anchored PCR using uncloned single-strand human testis cDNA with an anchor primer and a second primer that is specific to SBP. RACE products were electrophoresed, and DNA fragments were subcloned into PCR2 vectors for sequence analysis (Fig. 5A). Fifteen PCR

2B) and MA-10 cells (Fig. 2C). These findings reflect the interaction between SBP and STAR protein in vivo.

SBP mRNA Expression in Human Tissues—Northern blot analysis was performed to examine the expression of SBP. Northern blots that each contained 2 μg of poly(A)$^+$ RNA isolated from the indicated tissues were probed sequentially with SBP and β-actin cDNAs. Expression of the SBP gene was detected in all tissues examined. Its expression level was particularly high in the testis, where the sizes of transcripts were 2.4 and 3.8 kb (Fig. 3). To examine the expression of SBP in various cell lines, we performed RT-PCR using mRNA extracted from Hep G2 cells, KGN cells, H295R cells, and MCF-7 cells. The expected amplification products (400 bp) were obtained in all of the cell lines examined (Fig. 4).
Alternative splicing of the SBP gene is represented in the schematic (A). Fifteen RACE products were subcloned and were electrophoresed (A). PCR was performed, and RACE products were electrophoresed (A). RACE analysis using Marathon-Ready human testis cDNA. Anchored man SBP gene. The transcription start sites were analyzed by 5'-splice-site and 3'-polyadenylation sites interact with the same 3'-polyadenylation signal. Although SBP has exon 1, it has two transcription start sites from alternative splicing and SBP is localized in organelles, including endosomes and mitochondria, in the cytoplasm.

**Fig. 5. Transcribed starts site and alternative splicing of human SBP gene.** The transcribed start sites were analyzed by 5'-RACE analysis using Marathon-Ready human testis cDNA. Anchored PCR was performed, and RACE products were electrophoresed (A). Fifteen RACE products were subcloned and were electrophoresed (B). Alternative splicing of the SBP gene is represented in the schematic (C). Numbers represent exons, and solid black boxes represent coding regions. Alternative transcription of the two 5'-untranslated exons, exon 1a and exon 1b, are indicated in the diagram. The numbers of nucleotides indicate sizes of introns 1a and 1b. ATG indicates the first start site ATG in exon 2, exon 3, and exon 4, and T AA in exon 18 indicates the end of the coding region.

products were sequenced, and the sequences were compared with the human SBP genome sequence (Fig. 5B). The SBP gene has two transcription start sites from alternative splicing and several minor transcription start sites. Although SBP has exon 1a and exon 1b, both 5'-sites interact with the same 3'-splice acceptors (Table III). The main transcription start sites are located 79 (exon 1a) and 430 bp (exon 1b) upstream of the translation start site (ATG) in exon 2. The first translation start site ATG in clone 4 is present in exon 4 in SBP in the testis. Clone 4 encodes an N-terminally truncated 656-amino acid region of the HCR protein (757 amino acids) (28) (Fig. 5C). From the RACE results, we expressed an plasmid (pSBP) by inserting the EcoRI fragment of the coding region of 757 amino acids amplified by PCR from testis cDNA into the pTarget vector (Promega).

**Effect of SBP on Steroid Hormone Production—**The SBP gene was found to be expressed in steroid hormone-producing cells, including H295R cells and KGN cells. To examine the effect of the SBP on the action of StAR protein, COS-1 cells were cotransfected with F2, StAR, and SBP expression plasmids (pSBP). The amount of pregnenolone produced by COS-1 cells was increased by 138% compared with that produced by cells transfected by F2, pStAR, and an empty vector (Fig. 6). siRNA Treatment Decreases the Production of Steroid Hormones—To examine further the role of SBP in the production of steroid hormones in cells, we performed an experiment to eliminate SBP expression using siRNA (29) and assayed steroid hormones. Two target sequences of the SBP gene were selected for gene silencing. The effects of siRNAs were assayed by RT-PCR. SBP gene expression levels were reduced by both siRNA-SBP-I and siRNA-SBP-II. After treatment of cells transfected with the double-stranded RNAs, the amounts of pregnenolone produced by H295R cells (85 ± 5.0 and 66 ± 5.2 ng/dish when treated with siRNA-SBP-I and siRNA-SBP-II, respectively) were significantly less than (p < 0.05) that produced by H295R cells transfected with scramble siRNA (Fig. 7A). The amounts of pregnenolone produced by H295R cells treated with SBP-I and SBP-II siRNA were decreased by 56.5 and 37.5%, respectively, compared with the amount produced by H295R cells treated with scramble siRNA. The amounts of progesterone produced by KGN cells treated with SBP-I siRNA and SBP-II siRNA were also decreased by 71 and 55%, respectively, compared with the amount produced by KGN cells treated with scramble siRNA (Fig. 7B). Reducing the level of SBP gene expression by siRNA treatment of targeted SBP gene sequences resulted in a decrease in the production of steroid hormones.

**Determination of SBP Localization in Cells—**Fluorescent microscopic observations were performed to determine the subcellular localization of SBP in COS-1 cells that had been cotransfected with pSBP-GFP and pStAR-REP. Punctate cytoplasmic staining of SBP-GFP fusion protein was observed in cells. The size of punctate signals varied from one vesicle to another (Fig. 8A). StAR-REP fusion protein exhibited ovoid-shaped signals in a reticulum pattern. These signal patterns are consistent with the characteristics of mitochondria (Fig. 8B). SBP-GFP fusion protein signals were partially overlapped with StAR-REP fusion protein signals (Fig. 8C). The findings indicate that SBP is localized in organelles, including endosomes and mitochondria, in the cytoplasm.

**DISCUSSION**

We screened a human testis cDNA library to identify proteins that interact with the StAR protein in the cytoplasm or outer mitochondrial membrane. Three clones, Rab5EP, NUCB2, and HCR, were identified by the screening. NUCB2, which functions as a DNA-binding protein, is a novel DNA-binding/EF-hand/leucine zipper (NEFA) (30). StAR homology domains are known as StAR-related lipid transfer (START) domains (31). START domains have been found in many kinds of proteins, including transcription factors (31). NUCB2 may be a transcription factor that interacts with the START domain that StAR-like transcription factors have. Rab5, which is a protein belonging to the family of Rab GTPases, regulates sequential transport steps along the endocytic and recycling pathway on early endosomes in cells (32). The secondary structure of SBP was predicted to consist of α-helical coils, which are features of the cytoskeleton, and it was therefore named α-helical coiled-coil rod homologue (HCR) (28). A BLAST search revealed that the amino acid sequence of SBP has little homology with those of known proteins. SBP did not interact with a StAR protein mutant that lacked the biologically active C terminus of the StAR protein (33). Thus, SBP protein interacts with the START domain of the C terminus of the StAR protein in cells. In mammalian two-hybrid assays, the interaction between StAR protein and SBP in cells transfected with plasmids of reverse orientation (pM-SBP and pVP16-N-62-StAR) was less than that in cells cotransfected with the expression vectors (pM-N-62-StAR and pVP16-SBP). These fusion protein structures may restrict interaction between SBP and StAR protein in cells.

SBP was found to be expressed in all tissues and in all cell lines examined, including steroid-producing cells. However, the
expression level was not high except in the testis. After cotransfection with F2, pStAR, and pSBP, the activity of steroidogenesis was increased by 138%. The results of siRNA experiments also support the function of SBP, which have the effect of steroidogenesis in cells. Although the function of the larger transcription of SBP is not known, RACE results showed that SBP has several transcriptions start sites. Alternative transcription of the 5’/H11032-untranslated region can be regulated in a tissue by alternative usage of promoters. SBP may have a larger translated protein that acts as a pro-protein of SBP or may translate another protein that has a different function in the testis. Although many two-hybrid libraries contain partial rather than full-length cDNAs, small cDNA molecules may improve the sensitivity of the selection process (34). Clone 4 encodes an N-terminally truncated 656-amino acid region of SBP. Although clone 4 is supposed to be a partial cDNA, truncated SBP may function in the testis. Recently, StarD4, StarD5, and StarD6 proteins, which each contain a START domain, have been identified using cDNA microarrays. StarD4 and StarD5 are ubiquitously expressed, whereas StarD6 expression is limited to the testis. These proteins are thought to function in the intracellular shuttling of sterols or other lipids (35). It is possible that StAR protein and SBP not only function in steroidogenesis but also function together for the transport of cholesterol to regulate cellular metabolic processes, because StAR protein is present not only in Leydig cells but also in Sertoli cells in the testis (36).

Although steroidogenesis requires the continuous synthesis of new StAR proteins and is associated with the 37-kDa pre-protein (37), an N-62-StAR recombinant protein stimulates the transfer of cholesterol from sterol-rich liposomes to mitochondria (38). Based on its physical characterization, StAR protein changes its conformation into a molten globule to associate
with the outer mitochondrial membrane and causes the transfer of cholesterol to the inner mitochondrial membrane (39–41). These findings suggest that StAR protein functional sites of action are outside mitochondria and transfer cholesterol from the outer membrane to the inner membrane. The facility of N-62 StAR protein to transport cholesterol between membranes in a reconstitute system has been reported to be 1.8 molecules of cholesterol/molecule of the StAR protein (41). The facility in a reconstitute system is smaller than that in Y-1 cells, in which StAR protein is able to transfer up to 400 molecules of cholesterol (37). It is likely that another protein in the cytoplasm is required for effective cholesterol transport. The peripheral-type benzodiazepine receptor (PBR) has been shown to function in steriodogenesis, mitochondrial respiration, apoptosis, and cell proliferation (42, 43). Because the PBR has been found in the outer mitochondrial membrane, where StAR protein is thought to act (42), StAR protein and SBP may work together in steriodogenesis with supporting functions of PBR. Alternatively, SBP may bind StAR protein and sustain StAR protein outside of the mitochondrial membrane to stimulate steroid hormone production, because the function of StAR protein on the outer mitochondrial membrane is regulated by its rate of import into mitochondria (44).

Lipid droplets in the cytoplasm, in which steriodogenic cholesterol is stored, are tightly attached to intermediate filaments. Mitochondria are also attached to intermediate filaments (45). The transport of cholesterol to mitochondria is associated with filaments and regulates steriodogenesis (46). MLN64, which resides in late endosomes and lysosomes, is proteolytically cleaved and includes START domains (47–50). MLN64 has been reported to associate with the mobilization of low density lipoprotein-derived cholesterol through late endosomes to mitochondria. Observation under a confocal microscope revealed that MLN64 in late endosomal tubules and StAR protein in the microtubule-dependent mitochondrial matrix exist in close proximity but are not completely overlapped in cells (51). We have shown that signals of SBP, which is localized in organelles, including endosomes and mitochondria, in the cytoplasm are overlapped with StAR protein signals. SBP may interact with StAR protein in the cytoplasm. SBP has important functions with StAR protein in cells: one is steriodogenesis, as is well known, and the others are unknown. Further studies, including studies using mouse knockout models, are needed to determine the functions of SBP.

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