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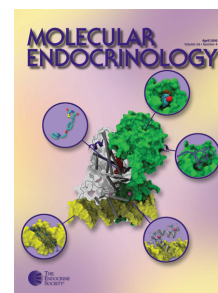
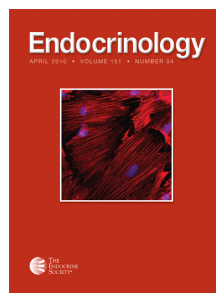
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Clinical, Genetic, and Functional Characterization of Four Patients Carrying Partial Loss-of-Function Mutations in the Steroidogenic Acute Regulatory Protein (StAR)

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Context: Nonclassic congenital lipid adrenal hyperplasia (lipoid CAH) is a recently recognized disorder caused by mutations in the steroidogenic acute regulatory protein (StAR) that retain partial function. Affected individuals can present with a phenotype of late onset adrenal insufficiency with only mild or minimally disordered sexual development.

Objectives: The aim was to delineate the clinical spectrum of StAR mutations and correlate phenotype with StAR activity.

Patients: Four patients had nonclassic/atypical lipoid CAH. Adrenal insufficiency was manifested at birth in two patients and at 11 months and 4 yr in the other two. Three were 46,XY with underdeveloped genitalia.

Methods: The StAR gene was sequenced, mutations were recreated in expression vectors, and StAR activity was measured as pregnenolone production in COS-1 cells cotransfected with the cholesterol side-chain cleavage system. StAR mutants were expressed as N-62 StAR in bacteria, and purified proteins were tested for activity with isolated steroidogenic mitochondria and for cholesterol-binding capacity.

Results: DNA sequencing identified mutations on all alleles. Missense mutations were R188C, G221D, L260P, and F267S; we also tested R192C described by others. The respective activities of R188C, R192C, G221D, L260P, and F267S were 8.0, 39.4, 2.4, 3.1, and 6.1% of wild-type in transfected cells, and 12.8, 54.8, 6.3, 1.8, and 9.5% with isolated mitochondria. Cholesterol binding capacities of R188C, R192C, G221D, L260P, and F267S were 6.7, 55.3, 10.2, 4.6, and 20.9%. These data are correlated to the three-dimensional structure of StAR.

Conclusions: There is a broad clinical spectrum of StAR mutations; StAR activities *in vitro* correlate well with clinical phenotypes. (*J Clin Endocrinol Metab* 95: 3352–3359, 2010)

Congenital lipid adrenal hyperplasia (lipoid CAH) is the most severe form of congenital adrenal hyperplasia (CAH) in which the synthesis of all adrenal and gonadal steroid hormones is impaired. Early clinical hormonal studies and incubations of affected tissue *in vitro* with various precursors identified a defect in the conversion of cholesterol to pregnenolone, so that the disorder was initially called “20,22 desmolase deficiency” because it was thought to result from a defect in the enzyme system converting cholesterol to pregnenolone. This enzyme was later identified as mitochondrial P450_{scc}. For a review of the early history of lipoid CAH, see Ref. 1. However, the P450_{scc} gene is normal in lipoid CAH (2, 3), and in 1995 it was found that lipoid CAH results from mutations in the gene encoding the steroidogenic acute regulatory protein (StAR) (4, 5). StAR facilitates the movement of cholesterol into mitochondria, where it is converted to pregnenolone by P450_{scc} (6). Patients with classic lipoid CAH usually present with adrenal failure and salt wasting, beginning within the first few months of life, and have female external genitalia irrespective of genetic sex (7). Until the description of nonclassic lipoid CAH in 2006, only one minimally virilized 46,XY female had been reported (8). Nonclassic lipoid CAH is a recently recognized disorder caused by StAR mutations that retain partial activity (9). Affected individuals can present with adrenal insufficiency resembling nonautoimmune Addison disease with only mildly disordered sexual development or normal development with hypergonadotropic hypogonadism (9, 10). More than 40 StAR mutations causing classic lipoid CAH have been described (4, 7, 8, 11), but very few partial loss-of-function mutations have been reported (8–10). We describe four patients with nonclassic or atypical lipoid CAH, identify their StAR mutations, and characterize the activities of the mutants to explore genotype/phenotype correlations.

Patients and Methods

Patient 1

A 46,XY phenotypic male from a consanguineous Thai family had an uneventful birth and had two febrile seizures during his first 2 yr of life, but no laboratory data are available. He was first evaluated at Chulalongkorn University Hospital at age 4 yr for progressive pigmentation. His older brother had had progressive hyperpigmentation and at 3 yr had fever, convulsions, and sudden unexplained death. The patient had descended testes, a stretched penile length of 3.5 cm and glandular hypospadias. Hormonal data (Table 1) showed unmeasurable cortisol unresponsive to cosyntropin, low dehydroepiandrosterone (DHEA) sulfate, and grossly elevated ACTH. Plasma renin activity (PRA) was 9.62 ng/ml/h (normal range, 1.0–6.5), aldosterone was 25.5 ng/dl (normal range, 3–35), and the electrolytes were normal, indicating a subtle defect in mineralocorticoid secretion. Ab-

TABLE 1. Clinical and biochemical characteristics of patients

	Patient 1 R188C	Patient 2 L260P/F267S	Patient 3 R188C/W250X	Patient 4 G221D	Patient 5 (Ref. 10) R192C
Country of origin	Thailand	United States	Switzerland	Nepal	Lebanon
Karyotype	46,XY	46,XY	46,XY	46,XX	46,XY
Age at investigation	4 yr	Newborn	3.5 months	12 yr	5 yr
Genitalia	Normal male, glandular hypospadias	Micropenis, small testes, severe hypospadias	Micropenis, cryptorchidism	Normal female	Normal male
Presentation	Pigmentation	Ambiguous genitalia	Pigmentation, ambiguous genitalia	Adrenal crisis	Pigmentation
Adrenal imaging	Normal US	Normal CT	ND	Normal MRI	ND
ACTH (pg/ml)	>1250	560	5497	2070	>225
Cortisol (μg/dl)					
Basal	0.1	8	5.3	<1	0.6
Peak	0.1	11	ND	ND	ND
PRA (ng/ml/h)	9.62	95	ND	15 mU/liter (3–40)	5.82
Aldosterone (ng/dl)	25.5	14	ND	<2.5	1.2
LH (IU/liter)	ND	15.2	7.6	ND	21.9
FSH (IU/liter)	ND	16.7	5.0	ND	50.4
Treatment	Prednisone	Hydrocortisone, fludrocortisone	Hydrocortisone, fludrocortisone	Hydrocortisone, fludrocortisone	Hydrocortisone

CT, Computed tomography; MRI, magnetic resonance imaging; ND, not done; US, ultrasound. Conversion to System International units: ACTH, picograms per milliliter × 0.22 for picomoles per liter; cortisol, micrograms per deciliter × 27.6 for nanomoles per liter; PRA, nanograms per milliliter per hour × 0.77 for picomoles per deciliter × 2.7 for picomoles per liter.

dominal ultrasound showed a normal-size right adrenal, but the left adrenal was not seen. The patient was treated with prednisone in doses equivalent to hydrocortisone 11–14 mg/m²/d and remained well with normal electrolytes. Sequencing of the ACTH receptor (MC2R), DAX1, and HSD3B2 genes was normal.

Patient 2

A 27-yr-old 46,XY male was born at term with micropenis, third-degree hypospadias, severe chordee, a hypoplastic scrotum, and palpable testes. A human chorionic gonadotropin stimulation test at 3–7 d of life failed to increase progesterone, 17-hydroxyprogesterone (17OHP), DHEA, androstenedione, and testosterone. His electrolytes were normal, and he did not require supplemental steroids during the neonatal period. At age 10 wk, cortisol was 8.5 μg/dl, but his ACTH was 560 pg/ml (normal, 10–50), indicating partially compensated adrenal insufficiency. His aldosterone was 14 ng/dl (normal, 5–90), and his PRA was 95 ng/ml/h (normal, 2.3–37). During an insulin tolerance test, his basal cortisol was 21 μg/dl, but it failed to rise further; an ACTH stimulation test failed to increase urinary 17-ketosteroids or serum levels of progesterone, 17OHP, cortisol, and DHEA. An abdominal computed tomography scan at age 12 wk did not show adrenal enlargement. The patient was diagnosed with “partial 20,22 desmolase deficiency” and started on lifelong glucocorticoid and mineralocorticoid replacement therapy. During his first 2 yr, he had several adrenal crises, including at least one with documented salt-wasting (Na, 124 mEq/liter; and K, 6.1 mEq/liter), associated with respiratory, gastrointestinal, or skin infections and requiring brief hospitalizations for parenteral glucocorticoids. He had normal growth and development and underwent a two-stage genital repair at age 2. He did not undergo puberty, and testosterone treatment was initiated during adolescence. When seen at the University of Pennsylvania at age 25, he was 185 cm tall, weighed 157 kg, and had hyperpigmented skin, 5-ml testes bilaterally, and a penile length of 4 cm. His 0800 h cortisol before taking hydrocortisone was less than 5 μg/dl, with ACTH 5911 pg/ml, total testosterone 78 ng/dl, LH 15.2 IU/liter, and FH 16.7 IU/liter. The parents declined genetic testing. His sister is unaffected, and her carrier status is unknown.

Patient 3

A 46,XY Serbian male infant presented at age 3.5 months having had prolonged neonatal jaundice. He had severe generalized hyperpigmentation, cryptorchidism, micropenis, but no hypospadias. When evaluated at University Children’s Hospital, Zurich, Switzerland, his sodium and potassium were normal, cortisol was 5.3 μg/dl, and ACTH was 5497 pg/ml. Aldosterone and PRA were not measured; testosterone was 29 ng/dl (normal

up to 232 ng/dl). He was treated with hydrocortisone and fludrocortisone and received three testosterone injections (25 mg) between 4 and 6 months of age with good penile response to 3.5 cm. He underwent right orchiopexy at 2 yr. When he was 8.5 yr old, his height was 134.5 cm (50th–75th percentile), and weight was 39.8 kg (>97th percentile) while receiving hydrocortisone 20.8 mg/m²/d and fludrocortisone 0.1 mg/d. He remained well with normal electrolytes but had a history of salt-craving. Sequencing of DAX1 and SF1 was normal.

Patient 4

A 15-yr-old, 46,XX Nepalese girl had recently moved to the United Kingdom and was referred to the Portsmouth Hospital with a diagnosis of “CAH”. She had first presented at 11 months of age with adrenal crisis including vomiting and dehydration and had subsequently been treated with hydrocortisone and fludrocortisone, but no hormonal data are available. She reported menarche at 12 yr of age and regular menstruation, but progesterone at d 22 of the cycle was low at 0.2 nmol/liter, indicating anovulatory cycles. Her ACTH was 2070 pg/ml, and she had unmeasurable cortisol (<1 μg/dl), aldosterone (<2.5 ng/dl), testosterone (<10 ng/dl), 17OHP (<60 ng/dl), androstenedione (<30 ng/dl), and DHEA sulfate (<15 μg/dl) (Table 1). Her plasma and urine steroid profiles were compatible with complete adrenal insufficiency. A magnetic resonance imaging scan showed normal-sized adrenals.

Mutation analysis, mutagenesis, and transfection

With informed consent, leukocyte genomic DNA was extracted, and the seven exons of the StAR gene were amplified by PCR and sequenced using previously described oligonucleotides and amplification conditions (12). Mutant full-length StAR cDNA expression vectors (13) were generated by PCR-based, site-directed mutagenesis (primers are shown in Table 2) and verified by direct sequencing. The PCR conditions were: 95 C for 30 sec, 16 cycles of 95 C for 30 sec, 55 C for 1 min, and 68 C for 18 min. Nonsteroidogenic monkey kidney COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37 C in a humidified 5% CO₂ incubator. Cells were divided into 12-well plates (Falcon; BD Biosciences, Lincoln Park, NJ) and cotransfected using Effectene (QIAGEN, Valencia, CA) at approximately 50% confluence. Cotransfections were done with a pCMV-StAR expression vector and the F2 plasmid expressing a fusion protein of the cholesterol side-chain cleavage system (H₂N-P450_{scc}-adrenodoxin reductase-adrenodoxin-COOH) (14). To monitor transfection efficiency, cells were also cotransfected with 5 ng of *Renilla* luciferase reporter plasmid (pRL-CMV) (Promega, Madison, WI) per well. Culture media were collected 48 h later, and pregnenolone production was measured by enzyme immunoassay (ALPCO Diagnostics,

TABLE 2. Oligonucleotide sequences for site-directed mutagenesis

Name	Sequence	NCBI accession no.; location
R188C	TTTGTGAGCGTGT T GCTGTGCCAAGC	NM_000349; 826
R192C	GCGCTGTGCCAAG T GCCGAGGCTCCAC	NM_000349; 838
G221D	AGGGCGGAGCACG A TCCCACTTGCATG	NM_000349; 926
L260P	ATCAACCAGGTCC C GTCACAGACCCAG	NM_000349; 1043
F267S	ACCCAGGTGGATT C TGCCAACACCTG	NM_000349; 1064

Boldface and underlined bases indicate a nucleotide change. The numbering of nucleotides is based on the NCBI human StAR cDNA sequence NM_000349. The numbering of the amino acid residues is based on the NCBI human StAR protein reference sequence NP_000340.2.

Salem, NH). The sensitivity of this assay is 5.4 ng/dl. Data are presented as the mean \pm SEM for at least three independent experiments, each performed in triplicate.

Preparation of vectors and proteins and *in vitro* biochemical assays

Each StAR mutation was inserted into the sequence of N-62 StAR in the modified pTWIN1 in vivo expression plasmid (15). The plasmids were individually transformed into *Escherichia coli* BL21DE3. StAR expression was induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside at 28 C for 6–8 h. Bacteria were lysed by sonication and cleared of debris; the supernatant was loaded onto chitin-binding columns (New England Biolabs, Ipswich, MA), washed, and eluted according to the manufacturer's recommendations. The N-62 StAR was collected and dialyzed against 300 mM NaCl, 20 mM Tris (pH 7.5) (15, 16). Wild-type N-62 StAR was quantitated by the Bradford method, and mutant proteins were quantitated with a standard curve of wild-type N-62 StAR (Fig. 1). Mitochondria were prepared from mouse MA-10 Leydig cells as described (13). Purified N-62 StAR proteins (5 μ M) were added to mitochondria (5 μ g of Bradford protein) in 50 μ l of 125 mM KCl, 5 mM MgCl₂, 10 mM KH₂PO₄, 25 mM HEPES (pH 7.4), 250 ng/ml trilostane, 100 μ M GTP, and 10 mM isocitrate, and incubated for 1 h; conversion of mitochondrial cholesterol to pregnenolone was measured by enzyme immunoassay, as above.

Cholesterol binding assay

Cholesterol binding capacity was measured by mixing purified N-62 StAR proteins (1 μ M) in PBS with various amounts of fluorescent 22-(*n*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-chole-3 β -ol) (NBD-cholesterol) (Invitrogen, Carlsbad,

CA) in 96-well plates (final volume, 100 μ l). The samples were incubated 1 h at 37 C and excited at 480 nm, and steady-state fluorescence emission at 540 nm (cutoff, 515 nm) was monitored with a SpectraMax M2 microplate reader (Molecular Devices Inc., Sunnyvale, CA) (16).

Results

StAR mutations

Patient 1 was homozygous for the mutation 5033C>T, changing arginine 188 to cysteine (R188C) (NCBI genomic DNA reference sequence NG_011827.1, and StAR protein reference sequence NP_000340.2) (17, 18). Homozygous R188C was found in one of the initial patients with non-classic lipoid CAH (9) and in four families reported in Ref. 10. Patient 2 was a compound heterozygote for 6732T>C, changing leucine 260 to proline (L260P); and 6753T>C, changing phenylalanine 267 to serine (F267S). L260P was described in Swiss patients with classic lipoid CAH (19); F267S is a novel mutation. Patient 3 was compound heterozygous for R188C and tryptophan 250 stop mutation (W250X). Homozygous W250X was previously found in a Serbian 46,XY phenotypic female with classic lipoid CAH (20). Patient 4 was homozygous for the novel mutation 5780G>A, changing glycine to aspartic acid (G221D).

Functional studies of the StAR mutants in transfected cells

We studied the functional consequences of the four missense StAR mutants identified here and the mutation R192C recently found in a family with nonclassic lipoid CAH, but which had not been characterized functionally (10). We cotransfected nonsteroidogenic COS-1 cells with either wild-type or mutant StAR and a vector expressing the F2 fusion of the cholesterol side chain cleavage system (14) and compared the amount of pregnenolone produced. An empty vector was used as a negative control, and 22R-hydroxycholesterol, which bypasses the action of StAR and thus indicates the maximal enzymatic capacity of the P450_{scc} system (4, 7, 13) was added as a positive control. Using endogenous cellular cholesterol and cholesterol in the serum in the culture media as substrate, COS-1 cells expressing F2 and wild-type StAR made 1917 \pm 422 ng/dl of pregnenolone, whereas cells transfected with the empty vector produced a low level of pregnenolone (23 \pm 11 ng/dl), indicating the presence of StAR-independent steroidogenesis. The R188C mutant produced 175 \pm 8 ng/dl of pregnenolone, whereas the G221D, L260P, and F267S mutants generated 68 \pm 17, 82 \pm 23, and 138 \pm 16 ng/dl, respectively. By contrast, R192C produced substantially more pregnenolone at

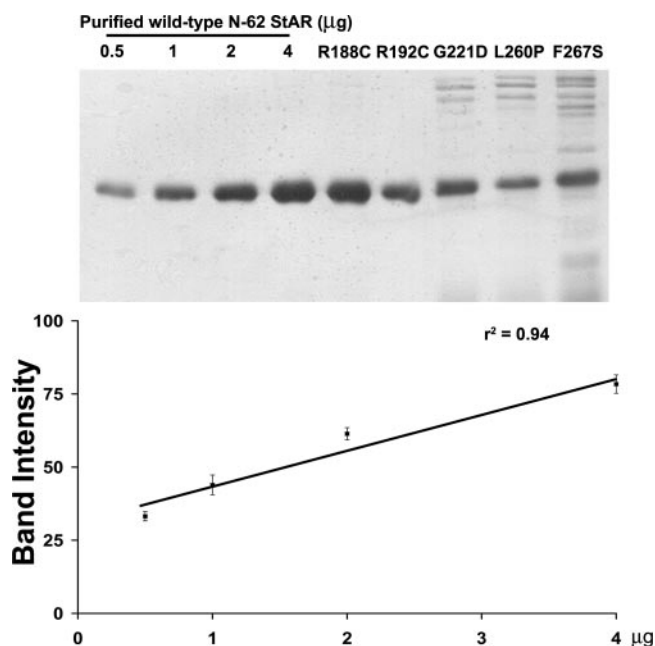


FIG. 1. Quantification of StAR protein. *Top panel*, The indicated amounts of purified wild-type N-62 StAR protein and mutant purified N-62 StAR proteins were separated by 12% SDS-PAGE. The band intensity was measured by Scion Image software. *Bottom panel*, The standard curve of StAR protein. The amount of mutant N-62 StAR proteins was plotted against its band intensity.

769 ± 169 ng/dl. When the background of StAR-independent steroidogenesis is subtracted, the R188C, R192C, G221D, L260P, and F267S mutants had 8.0, 39.4, 2.4, 3.1, and 6.1% of wild-type activity, respectively (Fig. 2A). In this assay, pregnenolone production in all five

StAR mutations was significantly higher than vector control ($P < 0.05$).

Activities of purified StAR proteins with steroidogenic mitochondria

To assess the activities of StAR mutants in a cell-free system, we expressed N-62 StAR protein using a self-splicing bacterial intein system. Purified N-62 StAR protein (Fig. 1) was added to mitochondria isolated from mouse Leydig MA-10 cells, and StAR activity was measured as pregnenolone produced from endogenous mitochondrial cholesterol. In this assay, wild-type N-62 StAR elicited 1816 ± 293 ng/dl pregnenolone, and the buffer control lacking StAR produced 263.3 ± 37.7 ng/dl pregnenolone, indicating a level of StAR-independent steroidogenesis approximately 14% of the StAR-induced level. After subtracting the buffer control, the R188C, R192C, G221D, L260P, and F267S mutants elicited 12.8, 54.8, 6.3, 1.8, and 9.5% of wild-type activity, respectively (Fig. 2B). In comparison to the buffer control, R188C ($P < 0.0001$), R192C ($P < 0.0001$), G221D ($P = 0.02$), and F267S ($P = 0.002$) elicited significantly more pregnenolone, whereas the activity of L260P was not different from the buffer paired (two-tailed Student's *t* test; $P = 0.57$).

Cholesterol-binding capacity of the mutant StAR proteins

Purified recombinant proteins were combined with various concentrations of NBD-cholesterol, and cholesterol binding was measured fluorometrically. The binding of fluorescent NBD-cholesterol to StAR is very similar to that of [¹⁴C]cholesterol; hence, this readily measured form of cholesterol is appropriate for measuring the cholesterol binding capacity of StAR (16). After subtracting the buffer control, the maximal cholesterol binding capacity of the R188C, R192C, G221D, L260P, and F267S mutants was 6.7, 55.3, 10.2, 4.6, and 20.9% of the wild-type value (Fig. 2C). Thus, the impaired activity of the mutants generally correlated with their impaired capacities to bind cholesterol.

Discussion

StAR moves cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane, thus providing the cholesterol substrate for steroidogenesis (4, 21). StAR exerts this action on the OMM (13, 22), where it must undergo a conformational change that results from its interaction with protonated OMM phospholipids (15, 23–25). The essential role of StAR in adrenal and gonadal steroidogenesis was established by finding recessive, loss-of-function mutations of StAR in

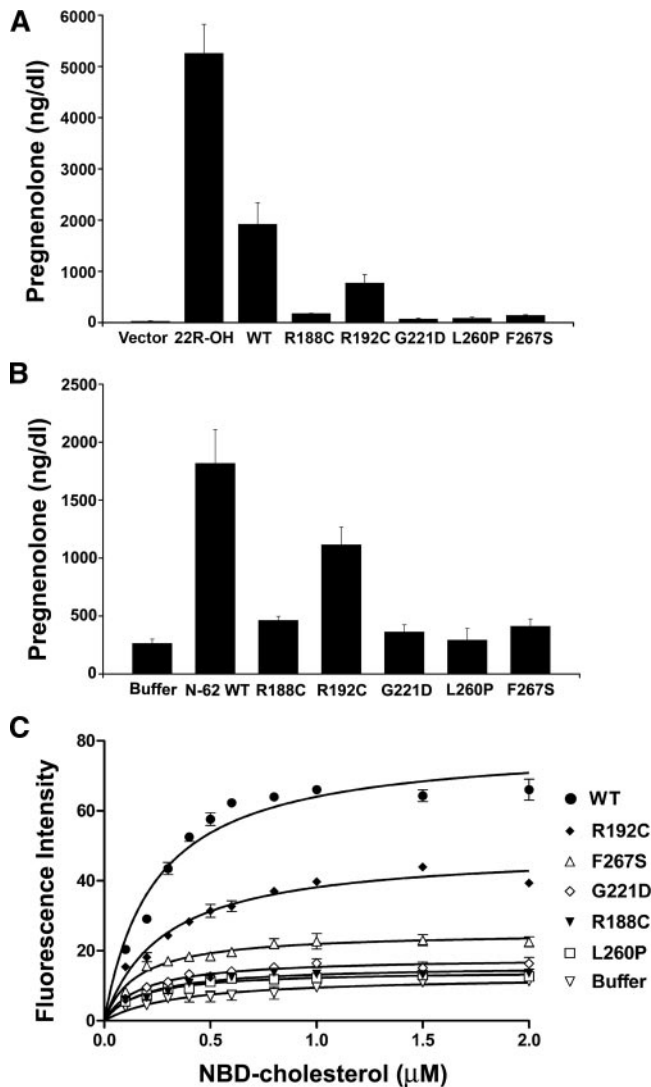


FIG. 2. Activities of StAR mutants. **A**, Activity of full-length StAR in whole cells. COS-1 cells were cotransfected with expression vectors for the cholesterol side-chain cleavage system (F2) and either wild-type (WT) or mutant StAR, and pregnenolone was measured 48 h later by immunoassay. The StAR-independent substrate 22(R)-hydroxycholesterol (22R-OH) was added to the cell culture media to determine the maximum steroidogenic capacity of the cells. Data are expressed as the mean ± SEM from at least three independent experiments, each performed in triplicate. **B**, Activity of isolated N-62 StAR on mitochondria *in vitro*. Purified proteins were added to mitochondria from steroidogenic mouse MA-10 Leydig cells, and pregnenolone production from endogenous mitochondrial cholesterol was measured. Data are expressed as mean ± SEM from four experiments, each performed in duplicate. **C**, Cholesterol binding assay. Binding of various concentrations of NBD-cholesterol by wild-type and mutant StAR was measured by fluorescence; control is buffer without protein. Data are expressed as mean ± SEM for three experiments, each performed in triplicate.

lipoid CAH (4, 5, 7, 8). Until recently, lipoid CAH was known exclusively as a very severe disorder of steroidogenesis characterized by greatly diminished or absent synthesis of all adrenal and gonadal steroids (26). Affected individuals with classic lipoid CAH are phenotypically female and have severe salt loss in early infancy. Nonetheless, affected 46,XX females spontaneously develop secondary sex characteristics and experience cyclical vaginal bleeding, but the cycles are anovulatory, and the patients have progressive hypergonadotropic hypogonadism later in life (12, 27). These unusual findings were explained by the two-hit model (7). In early lipoid CAH, StAR-independent mechanisms can still move some cholesterol into mitochondria, resulting in a low level of steroidogenesis, and increased corticotropin secretion (first hit). Over time, lipid droplets accumulate, damage the cell, and eventually destroy steroidogenic capacity (second hit). In the ovary, follicular cells remain unstimulated and thus undamaged until they are sequentially recruited in monthly cycles beginning at puberty. By contrast, the fetal testicular Leydig cells are affected early in gestation due to stimulation by chorionic gonadotropin.

In 2006, Baker *et al.* described three children from two families who presented with adrenal insufficiency at 2–4 yr of age; the males had normal genitalia. These patients were homozygous for StAR mutations V187M and R188C; functional studies of these mutants showed that they retained approximately 20% of wild-type activity (9). These data delineated a new disorder, nonclassic lipoid CAH. Later, Metherell *et al.* (10) reported five families with nonclassic lipoid CAH who had initially been misdiagnosed as having familial glucocorticoid deficiency. Four families carried R188C, and one carried R192C. All affected probands presented with Addisonian phenotypes at 2–7 yr of age, but one affected male remained undiagnosed and untreated until 58 yr of age (10). Several patients with nonclassic StAR mutations have progressive hypergonadotropic hypogonadism and azoospermia, potentially compromising fertility (10). Thus, identifying patients with nonclassic lipoid CAH is important because, unlike patients with familial glucocorticoid deficiency, they may have compromised fertility and mild salt loss.

Although massive adrenal enlargement is a classic hallmark of lipoid CAH (26), this sign is not pathognomonic. Small adrenals have been reported in patients with classic lipoid CAH (11), and such enlargement has not been reported in patients with nonclassic phenotypes (9, 10), although hypoplastic adrenals with calcifications, suggesting cirrhotic, end stage fat deposition, have been reported (10). None of the patients in our study had evidence of adrenal enlargement by abdominal imaging. Similarly, patients with CYP11A1 mutations causing P450_{scc} defi-

ciency, who have clinical manifestations that are indistinguishable from StAR mutations, have not been reported with enlarged adrenals (28). Thus, in the setting of adrenal insufficiency, massively enlarged adrenals indicate classic lipoid CAH; normal-sized adrenals cannot exclude it but instead suggest P450_{scc} deficiency or nonclassic lipoid CAH.

StAR mutations have been described in many ethnic groups but are common in Japan, Korea, and some isolated populations (1, 7, 8, 19). The R188C mutation found in our Thai patient has also been reported in patients from Canada, Jordan, India, and Pakistan (9, 10), suggesting a recurrent mutation. StAR mutations in intronic regions can also cause lipoid CAH (5, 29). Most StAR missense mutations are found in the carboxy-terminal 40% of the 285-amino acid StAR protein (1) and totally eliminate StAR activity. Only five previously described mutations were associated with residual StAR activity in transfected COS-1 cells: V187M (22%) (9), R188C (14%) (9), A218V (6%) (7), M225T (29%) (8), and L275P (10%) (7). A gain-of-function StAR mutation, Q128R, has been reported recently in a 46,XX phenotypic female infant who had salt loss at 4 months, but the coexistence of homozygous frameshift null mutation ablated all StAR activities, so the potential effect of the Q128R mutation was not evidenced in the patient (30). The mechanism by which this mutant gains function is unknown.

The manifestations and severity of disease differed substantially in our patients. Patient 1, who had the mildest phenotype, was homozygous for R188C. In our assays of intracellular activity, mitochondrial activity, and cholesterol-binding capacity, R188C retained 8, 12.8, and 6.7% of wild-type activity, respectively; when we studied this mutant previously, the values for these assays were 13.6, 17.7, and 21.0% (9). Hence, whereas there is some variability in these biochemical assays, it seems that 10–20% of activity will dramatically alter the classic phenotype. The report of the patient carrying R192C, which retained about 50% of wild-type activity, indicated even milder disease (10). By contrast, patient 2, carrying both F267S (~10% activity) and L260P (~3% activity) had fairly severe disease, as did patient 4, who was homozygous for G221D (~6% activity). Among the three assays we used, the direct assays of StAR activity, rather than the cholesterol-binding assay, appear to provide the best correlation with the clinical phenotype. A disparity between the activity assays and the binding assay is not surprising, because StAR mutation R182L was homozygous in patients with classic lipoid CAH and lacked activity in functional assays (7, 11) but retained normal capacity to bind cholesterol, indicating that cholesterol binding is necessary but not sufficient for StAR activity (16). Thus, there is a

fairly good correlation between StAR activity *in vitro* and the clinical phenotypes.

The crystal structures of three proteins closely related to StAR have been determined (31–33), permitting the construction of reliable computational models of human StAR (25, 34). StAR's structure features a hydrophobic pocket of the size and shape needed to bind cholesterol; the amphipathic C-terminal α -helix (C-helix) forms the "floor" of the pocket, with hydrophobic residues pointing inward and hydrophilic residues pointing outward. The structure shows no direct access to this pocket. Liposome protection experiments show that only the C-terminal α -helix (C-helix) interacts with the OMM (25), and the position of the C-helix is stabilized by a network of hydrogen bonds with the adjacent structures (25, 34). Molecular dynamics simulations indicate that interaction of StAR with protonated phospholipids on the OMM disrupts these hydrogen bonds, inducing a conformational change (molten globule transition), permitting the C-helix to swing open to allow the binding of cholesterol into the sterol binding pocket of StAR; consistent with this model, disulfide mutants that immobilize the C-helix but that do not alter StAR's conformation interfere with StAR activity and cholesterol binding (15, 35).

Many mutations that cause lipoid CAH, including those identified in this study, are located in the C-terminal helix or elsewhere in the sterol-binding pocket. Leu260 and Phe267 are conserved hydrophobic residues in the C-helix, forming part of the cholesterol-binding site. Arg188 and Arg192 lie in sheet β 6, and G221 is in the loop joining sheets β 8 and β 9, which contribute to the sterol-binding pocket. Consistent with this, the reduced activity of these mutants was associated with reduced cholesterol-binding capacity. The mechanism by which StAR facilitates the movement of cholesterol into the mitochondrion is incompletely understood, but it appears to involve several other proteins on the OMM. Functional data indicate that the 18-kDa peripheral benzodiazepine receptor (PBR; also called the mitochondrial translocator protein) must be present for StAR to act (36), and homo-bifunctional protein cross-linking data show that StAR comes into close contact with the voltage-dependent anion channel-1 and with a phosphate-carrier protein on the OMM (37). Additional data suggest that StAR participates in a large macromolecular complex, which, in addition to StAR, PBR, and voltage-dependent anion channel, includes PBR-associated protein 7, protein kinase A regulatory subunit I α , and adenine nucleotide transporter (38, 39). Substantial additional work is needed to delineate the role of each of these proteins in mitochondrial cholesterol import; the potential discovery of patients carrying mutations in any of these could be most informative.

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