BRIEF REPORT: THE MOLECULAR BASIS OF STEROID 5α-REDUCTASE DEFICIENCY IN A LARGE DOMINICAN KINDRED

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PSEUDOHERMAPHRODITISM in men is often caused by genetic deficiencies in the production or action of androgens.[1,2] One form of this condition is a deficiency of steroid 5α-reductase, the enzyme that catalyzes the conversion of testosterone to dihydrotestosterone in androgen-sensitive tissues.[3,4] Affected persons have 46,XY karyotypes. At birth, they have normal male structures derived from the wolffian duct, but defects in the external genitals ranging from simple hypospadias to a blind vaginal pouch and clitoris-like phallus. The most common defect is a urogenital sinus with a blind vaginal pouch.[1-4] During puberty, the affected boys undergo various degrees of virilization.[3] Affected females are normal.

In the past, many affected children were raised as girls until or after puberty, when they changed their sexual identity to male and lived as men.[5-8] More recently, with increasing recognition of the syndrome, the majority of affected persons have been raised as boys from birth[5,6]; this phenomenon has been particularly well documented in a large kindred living in several isolated villages in the Dominican Republic.[5-8] In this kindred, the disorder is inherited in an autosomal recessive fashion.[9]

Two 5α-reductase genes have been cloned. These genes encode different isoenzymes that can be distinguished on the basis of biochemical, pharmacologic, and genetic criteria.[10-13] The type 1 isoenzyme is encoded on chromosome 5, is optimally active at an alkaline pH, is expressed in low levels in the prostate, and is relatively insensitive to finasteride, a 4-azasteroid enzyme inhibitor.[10-12] The type 2 isoenzyme is encoded on chromosome 2, is optimally active at an acidic pH, is expressed in high levels in the prostate and many other androgen-sensitive tissues, and is sensitive to finasteride.[12-14] Male pseudohermaphroditism due to 5α-reductase deficiency is a consequence of mutations in the type 2 gene.[13,14] In this paper, we report the characterization of a mutation in 5α-reductase in a large kindred from the Dominican Republic and show that a change in a single base pair in exon 5 of the type 2 gene produces an enzyme with an altered optimal pH and an abnormal affinity for its cofactor, NADPH.

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METHODS

Genomic DNA Isolation

Skin fibroblasts obtained by biopsy from affected subjects were cultured in monolayers.[15] White cells were obtained from blood collected in tubes containing EDTA. Total cellular DNA was isolated with an Applied Biosystems model 340A nucleic-acid extractor.

Polymerase Chain Reactions

Individual exons of the 5α-reductase type 2 gene were amplified by the polymerase chain reaction (PCR) with thermostable DNA polymerases.[16] The locations and names of the exon-specific pairs of oligonucleotides are described elsewhere in detail.[14] Amplified DNA was subjected to PCR sequencing.[17] Analysis of conformation-dependent polymorphisms of single-stranded DNA was performed according to a modification[14] of the procedures of Orita et al.[18,19] This technique involves denaturing a portion of an amplified segment of double-stranded DNA by heating it in the presence of formamide. On denaturation, the single-stranded DNAs either rejoin to form the initial double-stranded DNA or fold back on themselves in different conformations, depending on their base composition and sequence. Electrophoresis on neutral polyacrylamide gels resolves the various reaction products into discrete bands. Fragments of DNA differing by as little as a single base pair form different single-stranded DNA molecules that can be resolved by electrophoresis.[18,19]

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis of a 5α-reductase type 2 complementary DNA (cDNA) was carried out as described previously.[20] The desired mutant was identified by direct DNA-sequence analysis and reconstructed into the starting expression vector,[13] according to standard methods of genetic engineering.

Expression Analysis in Embryonic-Kidney 293 Cells

Normal and mutant cDNAs were transfected into human embryonic-kidney 293 cells (American Type Culture Collection cell-repository line 1573) as described by Andersson et al.[13] The enzyme activity of 5α-reductase in lysates of transfected cells was characterized with the use of assays described previously.[13,20]

RESULTS

The Dominican Pedigree

Figure 1 shows an extended pedigree of the Dominican kindred; the 5α-reductase deficiency can be traced through seven generations. The pedigree includes only affected family members or those whose descendants were affected. Twenty-nine families with 47 affected male members are traceable to the original female carrier. Forty-four of these affected male subjects were born with a clitoris-like phallus, bifid scrotum, and urogenital sinus with a blind vaginal pouch.[3,6,8] One subject had penoscrotal hypospadias. In all subjects, the testes were in the abdomen, inguinal canals, or scrotal folds. Various degrees of virilization occur at puberty, including the development of a muscu-

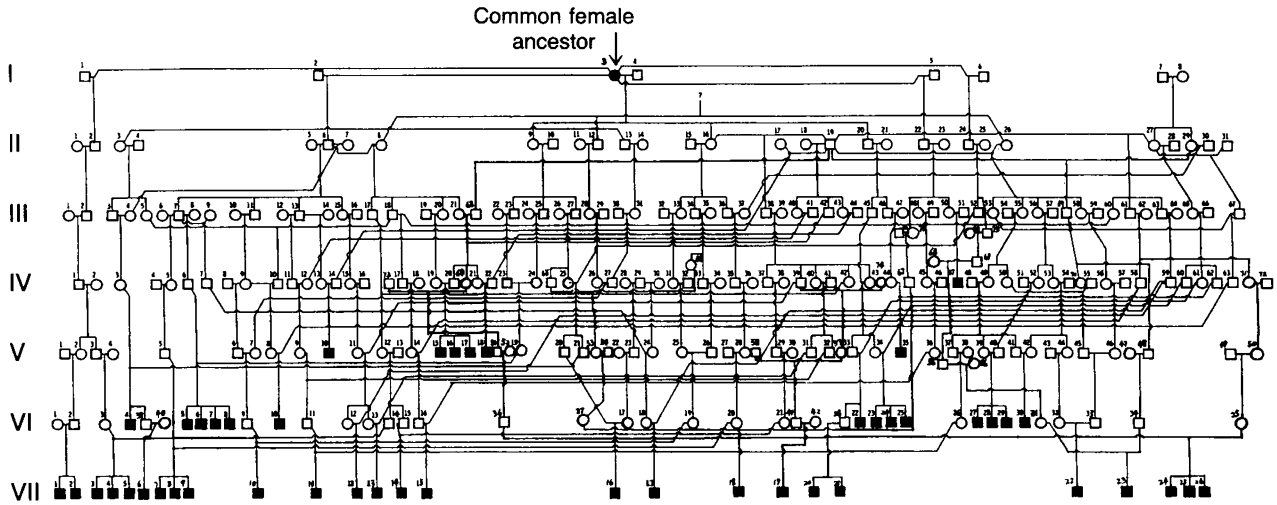

Figure 1. Seven-Generation Pedigree of the Dominican Kindred Affected by a Deficiency of 5α-Reductase Type 2.
Twenty-nine families share a common female ancestor in generation I, who had nine children with five partners. Couples in the six subsequent generations have had at least 47 affected male children (■), identified on the basis of family history, physical examination, endocrine testing, and in vitro biochemical assay. Circles denote female family members, and squares male family members.

lar habitus, deepening of the voice, and growth of the phallus. The subjects have markedly decreased amounts of facial and body hair in adulthood. Affected subjects have normal-to-elevated plasma testosterone concentrations and low plasma dihydrotestosterone concentrations, so that the ratio of testosterone to dihydrotestosterone in plasma is markedly elevated.[3,6,8] Decreased conversion of testosterone to dihydrotestosterone was confirmed by studies of enzyme activity in tissue and by analysis of urinary steroid metabolites.[21,22]

Identification of the Mutation in the Dominican Kindred

A point mutation in affected subjects was identified with analysis of conformation-dependent polymorphisms of single-stranded DNA (Fig. 2). No abnormalities were detected in the patterns of migration of single-stranded DNAs derived from exons 1, 2, 3, and 4 of two affected subjects (data not shown). However, analysis of exon 5 DNA from two affected brothers yielded a pattern of single-stranded DNAs that differed from that of normal subjects (Fig. 2A). The two brothers were homozygous for this pattern, whereas their parents were heterozygous carriers of the trait.

Analysis of genomic DNA from a number of affected subjects indicated that the abnormal pattern of exon 5 DNA was always present. This finding is illustrated in Figure 2B in three affected members of different families in the kindred. In addition, the pattern was found in a Dominican subject whose family has not yet been linked to the master kindred (Subject 506 in Fig. 2B). The strong correlation between the abnormally migrating single-stranded DNAs and the presence of the clinical syndrome suggests that the DNA polymorphism underlying the change in migration is the cause of the 5α-reductase deficiency.

DNA Sequence of the Mutation

The DNA sequences of exon 5 from a normal subject and an affected subject were determined. A difference of a single nucleotide was detected in the affected subject's DNA (a substitution of thymidine for cytosine) that gave rise to a tryptophan (TGG) in place of an arginine (CGG) at amino acid 246 (R246W) of 5α-reductase type 2. The substituted residue is located eight amino acids from the carboxy terminal of the protein. Analysis of the DNA sequence in affected subjects from three separate families, including Subject 506, revealed the same cytosine-to-thymidine mutation (data not shown).

Expression Analysis of the Mutation in the Dominican Kindred

To study the effects of the R246W substitution on 5α-reductase enzyme activity, the mutation was created in an expressible cDNA by site-directed mutagenesis. Normal and mutant cDNAs in eukaryotic expression vectors were transfected into human embryonic-kidney 293 cells, and enzyme activity was measured in whole cells and cell lysates (Table 1).

The normal 5α-reductase demonstrated a sharply acidic optimal pH of 4.9. In contrast, the mutant enzyme was found to have a more basic optimal pH (Table 1). When the activities of the normal and mutant enzymes were determined in cell lysates at their respective optimal pH values, the activity of the mutant enzyme was approximately 40 to 60 times less than that of the normal enzyme. The testosterone concentrations required to reach half-maximal

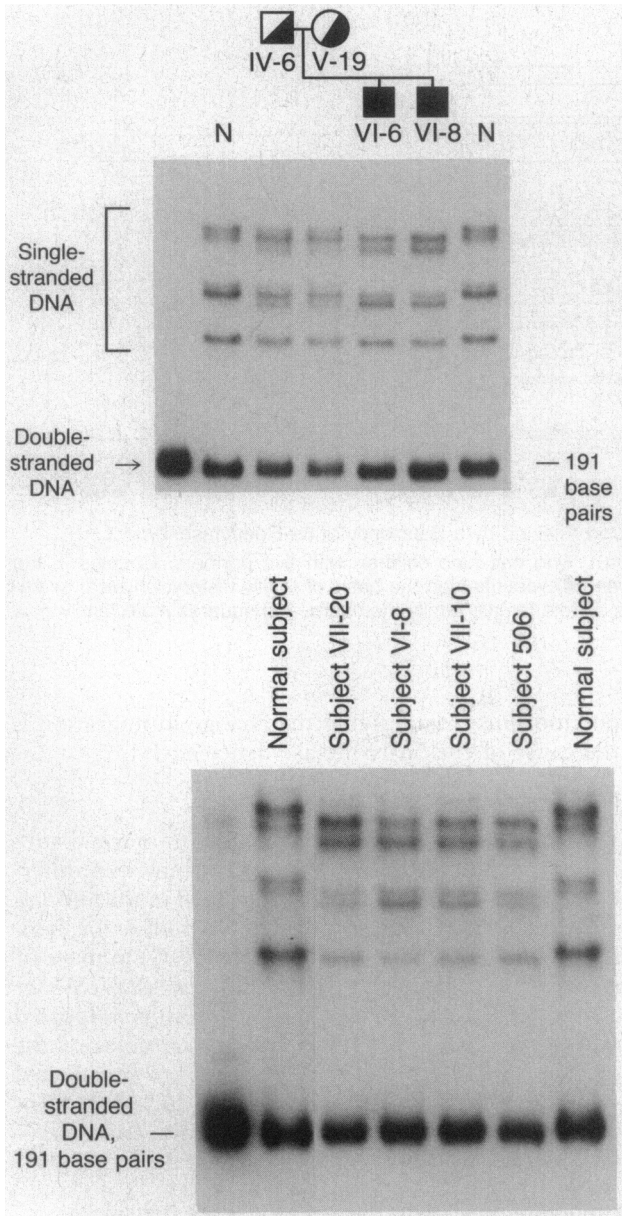

Figure 2. Inheritance of a Mutation on Exon 5 in a Dominican Family.

In Panel A, genomic DNA was extracted from Subjects IV-6, V-19, VI-6, and VI-8 and from a normal, unrelated subject (N), and exon 5 of the 5α-reductase type 2 gene was amplified by PCR. Radiolabeled amplified DNA was subjected to analysis on 5.4 percent neutral polyacrylamide gels and then to electrophoresis at room temperature. The dried gel was subjected to autoradiography with an intensifying screen for four hours at 22°C. The positions to which the double-stranded and single-stranded DNAs migrated are shown. The alterations in the migration patterns of the single-stranded DNAs are consistent with the carrier (semisolid symbols) and affected (solid symbols) genotypes of the subjects indicated in the pedigree. Squares denote male family members, and the circle a female member.

In Panel B, genomic DNA was extracted from a normal unrelated subject and Subjects VII-20, VI-8, and VII-10, and from fibroblasts of an affected Dominican subject not yet traced to the kindred (Subject 506). The DNA was analyzed for the conformation-dependent polymorphism of single-stranded DNA on exon 5, as described above. The gel was exposed to x-ray film for four hours at 22°C. The position to which the double-stranded exon 5 DNA (191 base pairs) migrated is indicated. The single-stranded DNAs of all affected members of the Dominican kindred migrate differently from those of the normal subjects.

velocity (apparent K_m [Michaelis constant] values) were similar for both the normal and mutant enzymes (Table 1).

The R246W mutation reduced 5α-reductase activity by decreasing the affinity of the enzyme for the NADPH cofactor. The normal enzyme expressed in embryonic-kidney 293 cells demonstrated saturation kinetics, and a concentration of 8 to 13 μmol of NADPH per liter was required to reach half-maximal velocity (Table 1). In contrast, the activity of the mutant enzyme increased almost linearly throughout the range of NADPH concentrations (0 to 25 mmol per liter) used in the assay. Lineweaver–Burk analysis of the results of multiple experiments indicated that the concentration of NADPH required for the mutant enzyme to attain half-maximal velocity was at least 600 μmol per liter (Table 1). In other experiments (data not shown), neither the mutant nor the normal enzyme could use NADH as a cofactor.

Discussion

We identified a missense mutation at amino acid 246 in the 5α-reductase type 2 gene of Dominican subjects with male pseudohermaphroditism. The cytosine-to-thymidine mutation in exon 5 of genomic DNA was present in homozygous form in a number of affected subjects. At the protein level, the mutation predicts a substitution of tryptophan for arginine in the carboxy-terminal region of the molecule. When assayed in lysates of transfected cells, the mutant cDNA reduced the activity of the enzyme, in agreement with previous findings in fibroblasts from these subjects in which low but measurable enzyme activity was detected.[22] The lack of complete feminization of the external genitals is consistent with the residual activity of approximately 6 percent measured in the transfected cells.

The finding of homozygosity in the affected subjects is predicted on the basis of the large number of consanguineous relationships in the kindred (Fig. 1) and biochemical detection of carrier status in numerous parents.[21] The presence of the same mutation in all the subjects and the high incidence of the disease in this kindred are almost certainly due to a founder-gene effect in this population, which is relatively isolated geographically.[9] The incidence of the disease was undoubtedly augmented by the large size of the families in the kindred.[9,23] The observation that 5α-reductase deficiency in females does not appear to decrease fertility, even in homozygous subjects,[6,8,9] also contributes to the high incidence of the disease.

Studies of male-sex identity in subjects with 5α-reductase deficiency from the Dominican Republic

Table 1. Biochemical Characterization of a Mutation of 5α-Reductase Type 2 in Affected Subjects.*

Variable	Normal Enzyme	Mutant Enzyme
Optimal pH	4.8–4.9	5.3–5.5
Maximal velocity of enzyme reaction (nmol dihydrotestosterone/min/mg of protein)	2.0–5.0	0.04–0.08
K_m† (μmol/liter)		
Testosterone	0.5–1.0	0.5–1.0
NADPH	8–13	600–650

*Values are the averages determined from duplicate points in at least two experiments carried out on different days with different cell lysates.

†K_m denotes the Michaelis constant, the substrate concentration at which the velocity is half-maximal.

and in other isolated populations have been used to gain insight into the role of androgens in establishing male patterns of behavior.[5] In the past, the abnormalities of external genitals at birth in these boys often led to their being raised as girls. A majority of subjects assumed a male pattern of behavior when their genitals enlarged, their voices deepened, and a male habitus developed during puberty.[5] This change in sexual identity suggested that exposure to testosterone, which is secreted in normal amounts in subjects with 5α-reductase deficiency, and not to dihydrotestosterone, whose levels are decreased, was the dominant factor and overrode rearing as a girl in determining male sexual identity.[5]

The conclusion that testosterone was responsible for masculinizing the brain was based on the assumption that there was only one gene for 5α-reductase in humans.[5,9] However, cloning studies have provided definitive evidence of two functional genes encoding different isoenzymes.[10-13] Of these, only the type 2 gene has been found to be defective in subjects with 5α-reductase deficiency.[12-14] The fact that two enzymes are present raises the possibility that dihydrotestosterone could be the key hormone in the masculinization of the brain.

The mutation in this Dominican kindred occurs in the cytosine of a cytosine–guanosine dinucleotide in exon 5 of the gene. Mutations involving cytosine–guanosine dinucleotides are common in the human genome[24] and are thought to be due to methylation-induced deamination of 5-methylated cytosines in this sequence. The cytosine that is mutated in the Dominican subjects may prove to be at a site in which the frequency of mutation is high.

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