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TWO NOVEL MUTATIONS IN THE VASOPRESSIN V2 RECEPTOR GENE IN PATIENTS WITH CONGENITAL NEPHROGENIC DIABETES INSIPIDUS

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Families with congenital nephrogenic diabetes insipidus were analyzed with regard to mutations in the vasopressin V2 receptor gene. Family 1 shows an X-chromosomal recessive inheritance of the disease over 4 generations. A patient from this family was found to have a T→A transversion at nucleotide 1095, predictive for a substitution of serine 167 (which is highly conserved among G-protein-coupled receptors), by threonine. Both the mutant and the normal allele were detected in the maternal genome. The patient's healthy brother was homozygous for the normal allele. The patient from family 2 showed a T→C transition at nucleotide 727, predictive for a substitution of leucine 44 by proline. Analysis of the maternal genome revealed homozygosity for the normal allele. Thus a *de novo* mutation seems to have occurred. The nature and site of the mutation in family 2 suggest that it is responsible for the patient's disease.

The antidiuretic action of arginine vasopressin (AVP) is mediated by the vasopressin V2 receptor (V2R) expressed in renal collecting duct epithelial cells. Agonist binding to the V2R stimulates adenylylcyclase via the G-protein G_S, leading to an intracellular rise of cyclic AMP. According to the shuttle hypothesis, cyclic AMP mediates the insertion of submembranously stored water channels into the apical membrane (1). This event causes a dramatic increase of water permeability and allows the reabsorption of water from renal collecting ducts.
Nephrogenic diabetes insipidus (NDI) is characterized by a resistance of the kidney towards

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Abbreviations: AVP, arginine vasopressin; V2R, vasopressin V2 receptor; NDI, nephrogenic diabetes insipidus; XNDI, X-chromosomal recessive nephrogenic diabetes insipidus; PCR, polymerase chain reaction.

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AVP (2). In most cases, the congenital form of the disesase shows an X-chromosomal recessive inheritance (XNDI). Following molecular cloning of the human V2R (3, 4), mutations in the V2R gene were found in XNDI patients (5-8), and carrier status, determined by linkage analysis, has been confirmed by DNA sequencing (9-11). No mutation in the V2R gene was found in an atypical case of (autosomal-recessive) NDI; instead, inactivating mutations of the AVP-sensitive water channel were detected (12).

Functional defects have been demonstrated for two V2R mutants associated with XNDI. The R113H mutant, with a predicted substitution of arginine by histidine in the first extracellular loop in close vicinity to the second transmembrane domain, displays reduced ligand binding as a main defect (13). The mutant R137W, with a predicted substitution of arginine by tryptophan in the second intracellular loop close to the second transmembrane domain, is unable to stimulate adenylylcyclase (14). These studies prove biochemically that the mutations are responsible for the clinical phenotype.

Here we report on two novel missense mutations found in patients with congenital NDI.

Materials and Methods

Patients

Two families (families 1 and 2) with congenital NDI were studied. They reside in different parts of Germany and are apparently not related. The diagnosis was based on the clinical symptoms present shortly after birth and the lack of increase of urine osmolality after administration of desmopressin.

PCR, cloning of PCR products, sequencing

Genomic DNA was extracted from whole blood with the Nucleon DNA extraction kit (Scotlab, Heidelberg, FRG). The V2R gene (2064 bp) was amplified by PCR with 500 ng of genomic DNA as template and oligonucleotides flanking the coding region. The sequences of the primers were: 5'-TCCTGGGTTCTGTGCATCCGTCTGTCTGAC-3' (sense primer, corresponding to nucleotides 7 to 36 in the genomic sequence; numbering according to the EMBL entry L22206) and 5'-CTACACCCAGCTCAGTGAGCTGAC-3' (antisense primer, corresponding to nucleotides 2047 to 2070). The amplification was performed in a final volume of 50 µl. The reaction mixture contained: primers (20 pmol of each), genomic DNA (500 ng), 200 µM dNTP's, 1 unit Taq-Polymerase (Appligene, Heidelberg, FRG), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% bovine serum albumin and 10 mM Tris-HCl (pH 9.0). Initial denaturation at 94°C for 4 minutes was followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 64°C for 1 min and extension at 72°C for 1 min 30 s with a final extension for 3 min at 72°C. The PCR products were directly cloned into the PCRII-vector (Invitrogen, Leek, Netherlands). Sequencing of double-stranded DNA in both directions was performed according to the dideoxy chain termination method with the Sequenase 2.0 kit (United States Biochemical Corp., Braunschweig, FRG). Two independently amplified PCR products were sequenced from each of the patients. A total of 14 sense and antisense primers was used for sequencing. Primer sequences are available from the authors on request.

Asymmetric PCR and direct sequencing of PCR products

A first amplification was performed as described above. For a second, asymmetric amplification, 2 µl of the first reaction mixture were transferred to a tube containing 48 µl of a reaction mixture with 50 pmol of the internal primer 5'-CCGTGAAGTATCTGCAG-3' (sense primer, corresponding to nucleotides 937-953) or the internal primer 5'-GCACATAGACGACCAC-3' (antisense primer, corresponding to nucleotides 1425-1440). Excepting the primers, the composition of the reaction mixture was identical to that above. Each reaction was performed in duplicate. Initial denaturation at 94°C for 30 s was followed by 30 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 1 min and extension at

72°C for 2 min with a final extension at 72°C for 4 min. The reaction mixtures of two tubes were pooled, extracted with chloroform, precipitated overnight with 1 volume of 7.5 M NH_4Ac and 2.5 volumes of 100% ethanol, and washed twice with 70% ethanol. Sequencing of single-stranded DNA was as described above.

Results and Discussion

Family 1 has a history for the disease over 4 generations (Fig. 1). A patient (IV_1, Fig. 1), his mother (III_4) and his healthy brother (IV_2) were available for the the present study. Polyuria in the family is quite severe. At 12 years of age, the patient passes 8 l of urine per day, and two of his maternal uncles (III_2, III_3) pass 25 l of urine per day. Females (carriers) also show symptoms. The patient's mother passes 5-8 l per day, and his maternal grandmother (II_2) and the grandmother's stepsister (II_1) reportedly "drank a lot". As no information concerning the patient's great grandparents (I_1 and I_2) is available, the origin of the disease remains elusive. It seems likely, however, that the great grandfather was affected by the disease, since two of his daughters (from different marriages, II_1, II_2 respectively) have polydipsic sons (III_1, III_2, III_3). The pedigree suggests an X-linked mode of inheritance with varying symptoms in female carriers, probably as a consequence of secondary X-chromosome inactivation (2).

In the case of the patient, analysis of cloned PCR products, comprising the 3 expressed exons and 2 introns (2 kb) of the V2R gene (see Methods), revealed a T→A transversion at nucleotide 1095 (numbering according to EMBL entry L22206), leading to substitution of serine 167 by threonine (S167T; Fig. 2). The predicted amino acid exchange takes place in the

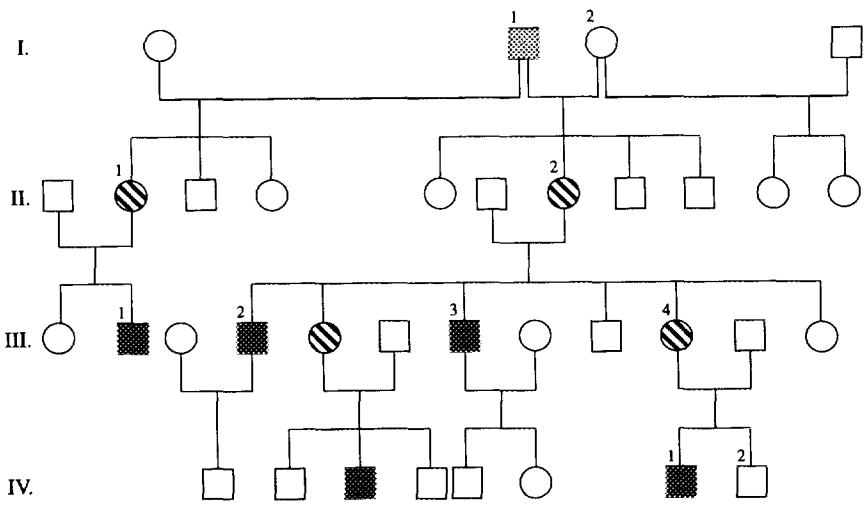

Fig. 1.
Pedigree of Family 1. Black squares and the grey square designate affected males and a presumably affected male, respectively. The hatched circles represent female carriers. Open symbols (squares, circles) designate unaffected family members, or family members, whose health state is either not known or not deducible. Except for the family members analyzed in the present study (IV_1, IV_2 and III_4), the pedigree is based on information obtained from the the mother (III_4) of the patient (IV_1).

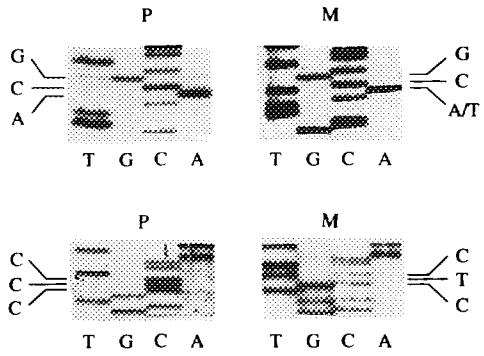

Fig. 2.
V2R gene sequences of family 1 (top) and family 2 (bottom). In the case of the patients (P), subcloned PCR products were sequenced. In the case of the patients' mothers (M), PCR products were amplified further by asymmetric PCR, and the single-stranded products were sequenced.

fourth transmembrane domain (Fig. 3). No other mutation was detected. Direct sequencing of single-stranded PCR products (see Methods) showed the presence of both the mutant and the normal allele in the maternal genome (see Fig. 2); only the normal sequence was detected in the genome of the healthy brother (not shown).

There are several reasons to assume that the S167T mutation causes NDI in family 1: (a) among the three family members analyzed, the mutant allele shows the distribution predicted for an X-linked recessive inheritance; (b) the exchange takes place in a region well conserved in G-protein-coupled receptors (15); at the corresponding position, a serine or alanine is found in 88 of 105 compared receptors; (c) a mutation at the same site (substitution of serine 167 by leucine) cosegregates with XNDI in four unrelated families (10, 11). Our data lend further evidence to the notion that Ser 167 is a "hot spot" for mutations (10). The importance of serine 167 for the function of the V2R (and of the corresponding residues in other G-protein-coupled receptors) remains to be elucidated.

Family 2 does not have a history for NDI. The only patient, who has no siblings, presented in a typical manner with mild fever, vomiting and polydipsia one week after birth. The diagnosis is based on the lack of increase of urine osmolality after intravenous application of desmopressin and high plasma levels of AVP. Under a drug regimen with hydrochlorothiazide and indomethacin, the urinary output of the patient (14 months of age) is 676 ml per 21 h.

Sequence analysis of of the patient's V2R gene revealed a T→C transition at nucleotide 727 (Fig. 2), leading to substitution of leucine 44 by proline (L44P). The exchange, creating a new restriction site for the enzyme Mnl I, takes place in the first transmembrane domain, in close vicinity to the extracellular N-terminus (see Fig. 3). No other mutation was detected. Direct sequencing of PCR products from maternal genomic DNA revealed the normal sequence only, indicating homozygosity for the normal allele (Fig. 2). Thus a *de novo* mutation in the maternal gametocyte might be responsible for the disease of the patient.

The L44P mutation is likely to be responsible for the disease. Hydrophobicity analysis

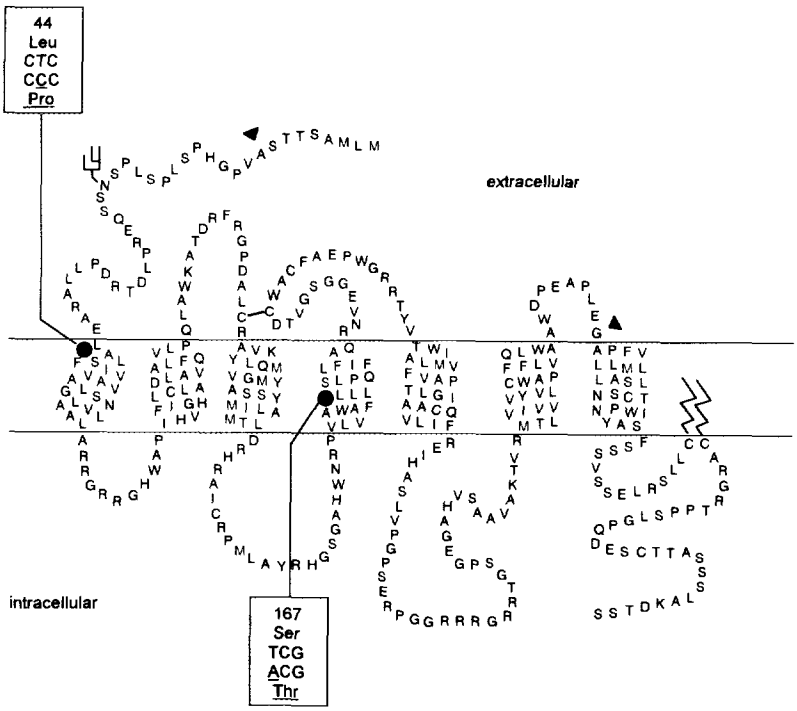

Fig. 3.
Predicted amino acid sequence and membrane topology of the human V2R. The depicted membrane topology is based on hydrophobicity analysis of the sequence (3). The one-letter code for amino acids is used. Arrowheads indicate exon/intron junctions (4). Based on the presence of consensus regions for post-translational modifications, the model depicts a sugar residue at asparagine 22 of the extracellular C-terminus, a disulfide-bridge between cysteines 112 and 192 of the first and second extracellular loops,respectively, and fatty acid residues at cysteines 341 and 342, attaching the intracellular C-terminus to the plasma membrane. The amino acids exchanged by the mutations described in the present paper (L44P, S167T) are shown as solid circles. The boxes depict the residue number of the exchanged amino acids, the amino acid in the wild type protein (three-letter code), the normal and mutated codons, and the amino acids in the mutant proteins.

according to Kyte and Doolittle (16) shows that the L44P change causes a decrease in hydrophobicity in the region involved. In addition, secondary structure analysis according to Garnier et al. (17) shows an extension of β-sheets and a corresponding reduction of α-helices at this site. Since proline residues introduce kinks into polypeptide chains, the mutation is predicted to cause a major conformational change. This is even more likely if one takes into account its location at the junction of the extracellular N-terminus and the first transmembrane domain (see Fig. 3).

Evidence for conformational changes induced by the introduction or removal of proline residues has been reported for mutants of another G-protein-coupled receptor, the light receptor rhodopsin (18). Three of these mutant proteins, which were found in patients with autosomal dominant retinitis pigmentosa, accumulate in the endoplasmic reticulum. Thus the transport of the L44P mutant to the plasma membrane may be impaired. Proof for this hypothesis by immunocytochemical experiments awaits the development of suitable V2R antibodies.

Acknowledgments

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