Human Hypertension Caused by Mutations in WNK Kinases


Hypertension is a major public health problem of largely unknown cause. Here, we identify two genes causing pseudohypoaldosteronism type II, a Mendelian trait featuring hypertension, increased renal salt reabsorption, and impaired K\(^+\) and H\(^+\) excretion. Both genes encode members of the WNK family of serine-threonine kinases. Disease-causing mutations in WNK1 are large intronic deletions that increase WNK1 expression. The mutations in WNK4 are missense, which cluster in a short, highly conserved segment of the encoded protein. Both proteins localize to the distal nephron, a kidney segment involved in salt, K\(^+\), and pH homeostasis. WNK1 is cytoplasmic, whereas WNK4 localizes to tight junctions. The WNK kinases and their associated signaling pathway(s) may offer new targets for the development of antihypertensive drugs.

A deletion in the linked interval. Additional loci in the completely linked telomeric interval were genotyped. One of these, D12S94, violated simple Mendelian transmission: all affected members were apparent homozygotes at this locus, and in all four informative matings, affected offspring inherited no allele from their affected parent (Fig. 1A). This finding indicates a null allele, consistent with a deletion within the disease-linked chromosome segment.

Genomic sequence of the interval containing this marker permitted identification of three additional polymorphic loci in close proximity to D12S94 (11) (Fig. 2A). Genotyping of these loci in K22 provided evidence of a null allele for each on the disease chromosome, with no evidence of transmission of alleles from affected parents to affected offspring (Fig. 1A). Flanking loci D12S341 and D12S91 showed no violations of Mendelian transmission. These findings indicate a deletion in the interval between D12S341 and D12S91.

We next determined endpoints of this deletion by Southern blotting of genomic DNA from kindred members, using the genomic sequence of the interval to define probes and restriction enzyme cleavage fragments for systematic screening (Fig. 2A) (12). A novel 6.9-kb fragment on the disease-linked chromosome was detected with two probes that are widely separated in wild-type genomic DNA (Fig. 2B). This result is consistent with a large deletion that encompasses the four loci showing null alleles by linkage (Fig. 2A).

Polymerase chain reaction (PCR) was used to amplify a product that spans the deletion endpoints (13). A primer pair normally separated by 42 kb in genomic DNA yielded a novel 600–base pair (bp) fragment when genomic DNA of affected, but not unaffected kindred members or 160 unrelated controls, was used as template (Fig. 2C). The DNA sequence of this fragment indicates that it arises from a deletion of 41,241 bp from normal genomic DNA (Fig. 2C). The two deletion endpoints occur within Alu repetitive elements (Fig. 2C). Use of other primer pairs confirms the presence of this deletion (14). Thus, a novel 41 kb genomic deletion cosegregates with PHAII in K22.

PHAII deletions lie in intron 1 of WNK1 and increase its expression. Analysis of genomic sequence (15) revealed that the deletion lies within the large first intron of the human ortholog of rat WNK1, a recently described serine-threonine kinase (16). This kinase is distinctive in having cysteine instead of lysine at a key position in the active site, and also contains two putative coiled-coil domains. The hWNK1 gene is encoded in 28 exons that span 156 kb in genomic DNA (Fig. 2D) and the encoded human and rat proteins are 86% identical in amino acid sequence. Transcripts of hWNK1 are found in

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: richard.lifton@yale.edu
most tissues (17), with two predominant length isoforms (Fig. 2E): a 10-kb transcript is expressed at high levels in the kidney, and a 12-kb transcript is predominant in heart and skeletal muscle.

One other PHAI1 kindred, K4, has shown linkage to 12p (8). All affected members share a haplotype across the 18 cM telomeric segment. Genotyping of loci across intron 1 of hWNK1 revealed null alleles for loci STS45K and STS60K (14). PCR using primers from this interval produced a novel 2.4-kb product, absent in 160 controls, that precisely cosegregated with PHAI1 in this kindred (Fig. 2F), and its DNA sequence revealed a deletion of 21,761 bp (Fig. 2F). The segment deleted in K4 is contained within the deleted segment of K22 (Fig. 2D). No deletions overlapping with this segment were detected in control subjects (18) and no mutations in the hWNK1 coding sequence were detected in these or 16 other PHAI1 kindreds (19).

To determine if the PHAI1-specific deletions alter the expression of WNK1, we compared WNK1 transcript levels in leukocytes from three affected members of K4 and three unaffected individuals (two from K4 and one unrelated individual) (20). The affected individuals had a fivefold increase in the level of hWNK1 transcripts (Fig. 2G), demonstrating that this intron 1 deletion alters hWNK1 expression.

Mutations in WNK4 on chromosome 17 also cause PHAI1. Additional PHAI1 loci have been mapped to chromosomes 17 and 1 (7). We searched genomic sequence and EST databases for paralogs of hWNK1 (15). Paralogs were identified on chromosomes 9 (hWNK2), X (hWNK3), and 17 (hWNK4). The encoded proteins all show high conservation in the kinase domain and have the distinctive substitution of cysteine for lysine in the active site (14). We localized hWNK4 to the interval on chromosome 17 between loci D17S250 and D17S379, both of which lie within the minimum genetic interval containing the PHAI1 locus on chromosome 17 (7). hWNK4 is encoded in 19 exons contained within 16 kb of genomic DNA (15) (Fig. 3A). The encoded amino acid sequence of hWNK4 shows 76% identity to hWNK1 across a 370 amino acid segment spanning the kinase domain and the first putative coil domain, 51% identity across an 83 amino acid segment encompassing the COOH-terminal putative coil domain, and 52% identity across a 102 amino acid segment from residues 640 to 741 of WNK4 (Fig. 3B and Web fig. 1) (21). The intron-exon boundaries within these domains are conserved between the two genes (Web fig. 1) (21). WNK4 is expressed virtually exclusively in the kidney (Fig. 3C) (17).

Examination of WNK4 in PHAI1 kindreds (19) identified four missense mutations, all of which cosegregated with the disease (Fig. 4). Three of these are charge-changing substitutions that cluster in a span of four amino acids just distal to the first putative coil domain (Fig. 4, A through C). These all lie within a negatively charged 10 amino acid segment that is highly conserved among all members of the WNK family in humans (Fig. 4F) as well as their known orthologs in mouse and rat (22). For example, in K13 (23, 24), a kindred previously linked to chromosome 17 (7), all eight affected members, but none of the unaffected members, inherit a single base substitution that produces a Glu→Glu substitution in WNK4 (Fig. 4, A and C). Similarly, mutations Asp→Glu→Ala and Glu→Lys were identified in affected members of K23 (25) and K11 (26, 27), respectively (Fig. 4, B and C). A fourth mutation, in PHAI1 kindred K21 (28), lies just distal to the second putative coil domain, and also changes charge, mutating Arg1185→Cys at a residue conserved among WNK4, 1, and 2 (Fig. 4, D and E). None of these mutations were identified among 140 unrelated unaffected control subjects. WNK1 and WNK4 localize to the distal nephron. To explore the expression patterns of WNK1 and WNK4, we prepared affinity-purified polyclonal antibodies that show specificity for each protein (Web fig. 2) (21, 29). Immunofluorescence microscopy of mouse kidney sections (30) demonstrated that both proteins localize to the distal convoluted...
tubule (DCT) and the cortical collecting duct (CCD), adjacent segments of the distal nephron that play a key role in salt, water, $K^+$, and pH homeostasis (Figs. 5 and 6). WNK1 was also abundant in the medullary collecting duct; neither protein was detected elsewhere in the kidney (Web fig. 3) (21). WNK1 and WNK4 show distinct subcellular distribution patterns. WNK1 is present throughout the cytoplasm (Fig. 5) whereas WNK4 is present exclusively in intercellular junctions in the DCT and in both the cytoplasm and intercel-

Fig. 2. Characterization of deletions in WNK1 in PHAII. (A) Structure of wild-type (WT) and deleted alleles on 12p in K22 (38). Polymorphic sequence tagged sites (STSSs) are indicated above the horizontal line representing genomic segments, and cleavage sites for PvuII are indicated below. The locations of probes used for Southern blotting in (B) and the sizes of resulting fragments are indicated. (B) Identification of deletion endpoints by Southern blotting in K22. Southern blots hybridizing probes from (A) to genomic DNA digested with PvuII are shown (12). Affected individuals of K22 are indicated by asterisks. (C) PCR across deletion endpoints in kindred K22. Products of PCR using primers separated by 42 kb in normal genomic DNA (13) are shown for members of K22 (M denotes marker lane); a 600-bp fragment cosegregates with PHAII. Below, the DNA sequence of a portion of the PCR product is compared to the sequence of the wild-type segment (WT). Numbered bases correspond to positions on the BAC clone in GenBank accession AC004765. The PCR product arises from a deletion whose endpoints fuse sequences normally separated by 41 kb. The deletion endpoints occur within a 32-bp repeated sequence (underlined). (D) Genomic structure of hWNK1. The genomic segment spanning WNK1 is represented by a horizontal line, and exons are indicated by numbered vertical lines. The genomic segments that are deleted in K22 and K4 are indicated. (E) Northern blot of hWNK1. A probe from hWNK1 was hybridized to RNA from a variety of human tissues (17). Locations of size standards in kilobases are indicated. (F) PCR across deletion endpoints in PHAII kindred K4. Primers separated by 24 kb in normal genomic DNA directed PCR from genomic DNA of members of K4; a 2.4-kb product cosegregates with PHAII. The DNA sequence demonstrated a 21.8-kb deletion, with the deletion endpoints in a 16-bp repeated sequence (underlined). (G) Increased levels of hWNK1 transcripts in PHAII. Quantitative RT-PCR (20) was used to compare the levels of hWNK1 and GAPDH transcripts in leukocytes from affected members of K4 (filled symbols) and control subjects (two unaffected members of K4 and one normal control subject; unfilled symbols). The mean and extreme values of repeated measures of the ratio of WNK1:GAPDH for each subject are shown.
Fig. 3. Characterization of hWNK4. (A) Genomic structure of hWNK4. The genomic segment spanning WNK4 is represented by a horizontal line, and exons of the gene are indicated by numbered vertical lines. (B) Comparison of hWNK1 and hWNK4. Domains of each protein are shown and the percentage amino acid identity (ID) between similar segments is indicated (21). Putative coil domains were predicted using the COILS program (39). (C) Northern blot of hWNK4. A probe from hWNK4 was hybridized to RNA from a variety of human tissues (17). Locations of size standards in kilobases are indicated.

Fig. 4. Missense mutations in hWNK4 in PHAII. (A) Mutation in exon 7 of hWNK4 segregates with PHAII in K13. Products of SSCP from exon 7 are shown in members of K13 (19). A novel variant (indicated by arrow) cosegregates with PHAII. (B) Mutations in exon 7 of hWNK4 in PHAII kindreds K11 and K23. Exon 7 was amplified as in (A). Affected members are indicated by asterisks and show novel variants (arrows) not seen in normal subjects (N). (C) DNA sequence of mutations in exon 7. The top panel shows the wild-type (WT) DNA sequence for codons 560 through 566 of hWNK4; the encoded amino acid sequence is shown above. In lower panels, the sequences of the variants identified in (A) and (B) are shown. Mutations are indicated by asterisks and the altered amino acids are shown in red. (D) Mutation in exon 17 in PHAII kindred K21. Exon 17 was amplified and fractionated as in panel A. The three affected members of K21 (asterisks) show a novel variant (arrow). (E) DNA sequence of mutation in exon 17. The WT DNA sequence for codons 1182 through 1188 of hWNK4 is shown at the top, and the mutant sequence in K21 is shown below. Conservation of residues mutated in PHAII among WNK family members. An 18 amino acid sequence of paralogous segments of hWNK1-4 is shown (40). An acidic 10 amino acid segment is highly conserved among all WNK family members. The mutations found in PHAII kindreds, indicated in red, alter completely conserved residues.

Fig. 5. Localization of WNK1 in kidney. Frozen mouse kidney sections were stained with antibodies and analyzed by fluorescence microscopy (29, 30). (A) Low-power view of renal cortex stained with anti-WNK1 (red) and anti-aquaporin-2 (AQP2, green), a marker of the connecting tubule and collecting duct. All tubules staining for AQP2 also stain for WNK1. In addition, other tubules in the cortex are also stained (DCT, see below). (B) Transverse section of cortical collecting duct (CCD) showing co-staining with anti-WNK1 and anti-AQP2. (C) Same view as (B) showing only anti-WNK1 channel, and demonstrating cytoplasmic distribution of WNK1. (D) Transverse section of distal convoluted tubule (DCT) stained with anti-WNK1 (red) and antibody to the thiazide-sensitive sodium chloride cotransporter (NCCT, green), an apical marker of the DCT. All tubules staining for NCCT also stain for WNK1. (E) Same view as (D) showing only anti-WNK1 channel. White bars represent 10 μm.
lular junctions in the CCD (Fig. 6, A through E). WNK4 colocalizes with ZO-1, a known tight junction protein (Fig. 6, F through H) but not with vinculin, an adherens junction protein (14). Thus, WNK4 is part of the tight junction complex. This finding is intriguing, as the tight junction is known to be the barrier to paracellular ion flux (31) and mutations in components of the tight junction have been shown to alter specific paracellular ion fluxes (32).

In summary, our findings establish that mutations in two members of the WNK family of serine-threonine kinases cause PHAII, with the evidence strongly supporting a genetic gain-of-function mechanism. The clustering of WNK4 mutations in a highly conserved domain suggests that they disrupt an interaction at this site required for the normal regulation of WNK4 function. Both kinases localize to distal nephron segments known to play a key role in the homoestasis of electrolytes altered in PHAII (Web fig. 4) (21). In the DCT, salt reabsorption is mediated by the electroneutral Na-Cl cotransporter. In the CD, Na⁺ is reabsorbed by the epithelial sodium channel (ENaC), with this electrogenic step providing the electrical drive for paracellular Cl⁻ flux in the CD (33).

Another possibility is that the mutant kinases cause constitutive activity of the electroneutral Na-Cl cotransporter in the CD, or a marked increase in its activity in its native site, the DCT. Identification of upstream regulators and downstream targets of these kinases will prove important in understanding the WNK signaling pathway(s).

The broad expression of WNK1 suggests that it may play a role outside the kidney, in which case missense mutations like those seen in WNK4 might prove lethal. Alternatively, different WNK1 isoforms might have different functional roles which restrict the types of mutation that can produce the PHAII phenotype. Linkage of PHAII to chromosome 1 in other families (7) as well as the absence of mutations in WNK1 and WNK4 in many PHAII kindreds indicate that there must be one or more as yet unidentified PHAII genes.

Like patients with PHAII, many individuals with common, so-called “essential” hypertension have low plasma renin activity and respond well to thiazide diuretics, raising the possibility that variants in WNK1 and WNK4 may underlie blood pressure variation in the general population. Indeed, the WNK4 gene lies only 1 Mb from locus D17S1299, the site showing the strongest linkage to blood pressure variation in the Framingham Heart Study population (34); this same chromosome segment has suggested linkage to hypertension in two other studies (35, 36). It will be of interest to determine whether variants that alter WNK4 function account for this linkage. Finally, WNK kinases and associated components of their signaling pathway(s) in the kidney may represent new targets for the development of antihypertensive diuretic drugs.

References and Notes
9. Members of K22 were classified as affected based on K⁺ > 5.0 and normal glomerular filtration (seven members), or a prior diagnosis of PHAII with current thiazide diuretic treatment (two members); in addition, a 12-year-old was classified as affected based upon K⁺ at the upper limit of normal, elevated blood pressure, suppressed plasma renin activity, hyper-chloremia, and reduced bicarbonate level. Kindreds studied included two showing definitive linkage to chromosome 12, one linked to chromosome 17, and 16 that are too small to prove linkage to any chromosome segment. Kindreds in which mutations were identified were Caucasian, ascertained in the United Kingdom (3 kindreds), France (2 kindreds), and Israel (1 kindred). Unrelated control subjects were Caucasians from the United States and Europe. The study was approved by the Yale Human Investigation Committee.
10. A genome scan was performed using 380 polymorphic markers spaced at 10-cM intervals. Lod scores were calculated (37) specifying PHAII as an autosomal dominant trait (disease allele frequency of 0.0001; penetrance 99%, phenocopy rate of 0.0001).
11. BLAST searches revealed that D12S341, D12S94, and D12S97 are on bacterial artificial chromosome (BAC) clone RPCI11-388A16 (GenBank accession AC004765). Three additional dinucleotide repeats were identified on this BAC and are designated STS42K, STS45K, and STS60K (Web table 1) (21). All are polymorphic; observed heterozygosities ranged from 0.30 to 0.60 in Caucasians.
12. Genomic DNA was digested with enzyme PvuII and Southern blotting was performed using [³²P]-labeled probes derived from the BAC clone. Probe 1 shown in Fig. 2A encompasses nucleotides 24593 through 25718 of WNK1.
The neutral hydrogen (H I) and ionized helium (He II) absorption in the spectra of quasars are unique probes of the intergalactic medium. By observing distant bright quasars in the early universe or from early epochs intermediate between the earliest density fluctuations seen in the cosmic background radiation and the distribution of galaxies visible today, the distribution of absorption features agrees to redshift (z) and the column densities of gaseous material in different ions reveal the structure of the IGM and its density and ionization state. From the ionization state of the gaseous species, we can also infer the processes responsible for ionizing the gas (e.g., radiation from quasars in the early universe or from early bursts of star formation).

The lack of smooth Lyα absorption by H

The neutral hydrogen (H I) and ionized helium (He II) absorption in the spectra of quasars are unique probes of the intergalactic medium. We present Far-Ultraviolet Spectroscopic Explorer observations of the line of sight to the quasar HE2347-4342 in the 1000 to 1187 angstrom band at a resolving power of 15,000. We resolve the He II Lyman α (Lyα) absorption as a discrete forest of absorption lines in the redshift range 2.3 to 2.7. About 50 percent of these features have H I counterparts with column densities $N_{\text{HI}} > 10^{12.3}$ per square centimeter that account for most of the observed opacity in He II Lyα. The He II is on H I column density ratio ranges from 1 to $>1000$, with an average of about 80. Ratios of $<100$ are consistent with photoionization of the absorbing gas by a hard ionizing spectrum resulting from the integrated light of quasars, but ratios of $>100$ in many locations indicate additional contributions from starburst galaxies or heavily filtered quasar radiation. The presence of He II Lyα absorbers with no H I counterparts indicates that structure is present even in low-density regions, consistent with theoretical predictions of structure formation through gravitational instability.

The intergalactic medium (IGM) is the gaseous reservoir that provides the raw material for the galaxies that dominate our view of the visible universe. By observing distant bright objects such as quasars, we can explore the IGM by examining the absorption features it

indicated antibodies, and subjected to immunofluorescence microscopy. WNK1 and WNK4 staining were both competed by the immunizing peptide (14).

8. The left and right ends of the segment shown lie at nucleotide positions 9575 and 107547 on BAC clone accession AC004765 in GenBank.
10. Amino acids for WNK2 and WNK3 are numbered with respect to GenBank entries reporting partial sequence for these genes (hWNK2, GenBank accession AB044456; hWNK3, GenBank accession A609088).
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