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Unveiling the distinct mechanisms by which disease-causing mutations in the Kelch domain of KLHL3 disrupt the interaction with the acidic motif of WNK4 through molecular dynamics simulation

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Abstract

Kelch-like 3 (KLHL3) is a substrate adaptor of an E3 ubiquitin ligase complex that regulates the degradation of its substrates, including with-no-lysine [K] kinase 4 (WNK4). Mutations in KLHL3 are associated with pseudohypoaldosteronism type II (PHAII), a hereditary form of hypertension. Many PHAII-causing mutations are located in the Kelch domain of KLHL3 that binds with WNK4; however, detailed mechanisms by which these mutations disrupt the binding are not well understood. In the present study we use molecular dynamics simulations and western blot analyses to examine the effects of these mutations on the interaction between the Kelch domain of KLHL3 and the acidic motif (AM) of WNK4. The simulation results correlated well with those from western blot analyses with the exception of the L387P mutation, which led to deregulation of AM degradation by KLHL3 but not recapitulated by simulations. Based on the simulation results, a mutation on the binding surface of the Kelch domain affected the Kelch-AM interaction through two major mechanisms: altering the electrostatic potential of the AM-binding site and disrupting the Kelch-AM hydrogen bonds. The mutations buried inside the Kelch domain were predicted by our simulations to have no or modest effects on the Kelch-AM interaction. Buried mutations R384Q and S410L disrupted intramolecular hydrogen bonds within the Kelch domain and affected

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SUPPORTING INFORMATION

This provides locations of the ten mutation-related residues on the Kelch domain of KLHL3 (Figure S1), comparison of the structural and dynamical properties between **WT** and **WT'** systems (Figure S2), secondary structural analysis for Kelch domain in WT and mutation systems (Figure S3), time-dependent RMSD of the Kelch-AM complex (Figure S4), dynamical cross-correlation map for mutant systems including S432N, S433N, R528H, N529K, R384Q, L387P, S410L and A494T (Figure S5), change of interaction containing E557 of WNK4 in **Q309R** system (Figure S6), change of hydrogen bonds containing D564 of WNK4 in **N529K** system (Figure S7), and comparison of the secondary structure and RMSF for residue 387 of Kelch domain between WT and L387P systems (Figure S8).

the Kelch-AM interaction indirectly. No significant effect of buried mutation A340V or A494T on the AM degradation or Kelch-AM interaction was observed, implying these mutations may disrupt mechanisms other than Kelch-AM interaction.

Graphical Abstract

Keywords

KLHL3; WNK4; hypertension; molecular dynamics simulation; PHAII-causing mutation

1. INTRODUCTION

Pseudohypoaldosteronism type II (PHAII), also known as familial hyperkalemic hypertension or Gordon's syndrome, is a hereditary disease characterized by hyperkalemia, metabolic acidosis, and hypertension (1, 2). Gene mutations in WNK (With-No-lysine [K]) kinases 1 and 4 (WNK1 and WNK4) were first identified in families of PHAII patients (3). WNK1 and WNK4 phosphorylate and activate oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK) (4–6), which in turn phosphorylate and activate the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC) and possibly other targets. In addition, WNK4 may phosphorylate NCC directly (7). PHAII mutations in WNK1 and WNK4 increase their protein abundance and/or kinase activity, and therefore raise NCC activity. Although effects of WNK kinases on other targets may also be involved, the clinical manifestation of PHAII can be explained by an increase in NCC activity alone, and thiazide diuretics, blockers of NCC, are very effective in managing PHAII.

Besides WNK kinases, mutations in two additional genes, Kelch like family member 3 (KLHL3) and Cullin 3 (CUL3), have been identified in PHAII patients (8, 9). KLHL3 and CUL3 are components of a cullin-RING E3 ubiquitin ligase complex, and recent research indicates that the substrates for this ligase complex include WNK kinases (10). In the ligase complex, CUL3 serves as a molecular scaffold that links the catalytic module and substrate adaptor, while KLHL3 acts as a substrate adaptor connecting both CUL3 and the substrate, and determines the specificity of the ubiquitin E3 ligase complex (11). So far only one type of PHAII mutation was identified in the CUL3 gene that generates a deletion in CUL3, which is likely to disrupt the interaction between CUL3 and KLHL3. By contrast, different PHAII mutations have been identified in many regions of KLHL3, including N-terminal BTB, BACK, and C-terminal Kelch domains. The mutations in the Kelch domain of KLHL3

disrupt the interaction with WNK1/WNK4 and those in the BTB and BACK domains disrupt the interaction with CUL3 (12).

The acidic motif (AM, ⁵⁵⁷EPEEPEADQ⁵⁶⁵) of WNK4 that harbors multiple PHAII mutations has been found to interact with the Kelch domain. Recently, the crystal structure of Kelch domain of KLHL3 in complex with AM of WNK4 has been deposited to the Protein Data Bank (PDB ID: 4CH9) (13). Similar to the other members of the BTB-BACK-Kelch family (14, 15), the Kelch domain of KLHL3 has a β -propeller structure which is formed by six Kelch repeats, each composed of four antiparallel β -strands (13). The AM of WNK4 is nested in the binding interface spanning all six Kelch repeats. So far at least ten disease-causing mutations (i.e. Q309R, A340V, R384Q, L387P, S410L, S432N, S433N, A494T, R528H, and N529K) have been identified in the Kelch domain (12, 13).

Interestingly, half of these mutations lie on the AM-binding surface and the other half are buried inside the Kelch domain. These mutations are expected to affect the interaction between WNK4 and the Kelch domain. However, it is unclear whether the mutations at the surface or inside the Kelch domain behave differently in affecting the interaction with AM of WNK4.

Recently, we utilized molecular dynamics (MD) simulation approach to study the effect of phosphorylation at S433 of KLHL3 on the interaction between Kelch domain and AM of WNK4 (Kelch-AM interaction) (16). The results from MD simulation are consistent with experimental results, indicating that MD simulation works well for the interaction. In the present study, to better understand the effects of the mutations on Kelch-AM interaction, MD simulations were performed with the structure of Kelch domain carrying different mutations. By structural, dynamical, electrostatic and interaction analyses, different effects of the PHAII-causing mutations on the interaction were determined. The distinct molecular mechanisms identified in this work may help understanding the molecular pathogenesis of PHAII.

2. MATERIALS AND METHODS

2.1 Western blot analyses.

The HA-tagged human KLHL3 and GST-tagged WNK4 acidic motif (peptide ⁵⁵⁷EPEEPEADQ⁵⁶⁵ of WNK4 fused to the C-terminal of GST tag) were used for western blot analyses. The KLHL3 mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the mutations were confirmed by sequencing. The capped complementary RNAs (cRNAs) of HA-tagged KLHL3 mutants were prepared using mMESSAGE mMACHINE™ SP6 Transcription Kit (ThermoFisher). The cRNA of WNK4 acidic motif with water, wild type KLHL3 or the KLHL3 mutants was microinjected in *Xenopus* oocytes at a concentration of 12.5 ng/oocyte. The animal protocol for *Xenopus* was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham. Then the lysates were extracted from ten oocytes for each group two days after cRNA injection. Western blot analyses were performed as described previously (17). Monoclonal anti-GST antibody (SC-138, 1:1000 dilution), anti-HA antibody (SC-805, 1:1000 dilution), anti- β -actin antibody (SC-47778, 1:1000 dilution), and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies

(1:10,000 dilution) were purchased from Santa Cruz Biotechnology. Chemiluminescence signals were detected using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL).

2.2 Simulation systems.

The structure of KLHL3 Kelch domain in complex with WNK4 AM was obtained from the Protein Data Bank (PDB ID: 4CH9) (13). The ten mutations, including Q309R, A340V, R384Q, L387P, S410L, S432N, S433N, A494T, R528H and N529N, were performed with the mutagenesis function of PyMol (18). Each mutation system was placed in a box filled with 11,570 transferable intermolecular potential 3P (TIP3P) water molecules (19). All the crystallographic waters were kept in the systems. The size of the water sphere was chosen so that the distance between every atom in the protein and the boundary of the water was at least 10 Å. Na⁺ and Cl⁻ ions were added to the system to neutralize it and maintain a 150 mM NaCl concentration. The Amber ff99SB force field (20) was applied to all the residues and ions including Na⁺ and Cl⁻ ions. For simplification, the simulation systems are denoted as *WT* system (containing wild type Kelch domain of KLHL3 in complex with AM of WNK4) and mutant systems (containing mutated Kelch domain of KLHL3 in complex with AM of WNK4, i.e. *Q309R*, *A340V*, *R384Q*, *L387P*, *S410L*, *S432N*, *S433N*, *A494T*, *R528H* and *N529N* systems).

2.3 Molecular dynamics simulations.

To investigate the effect of PHAII-causing mutations of KLHL3 on the interaction between KLHL3 and WNK4, ten 100 ns molecular dynamics (MD) simulations were performed for the mutation systems using the AMBER12 simulation package (21). The simulation procedure is the same to our previous work (22, 23). Each system was first relaxed by 2,000 steps of steepest descent followed by 2,000 steps of conjugate gradient minimization. After energy minimization of the whole system, the water molecules were equilibrated for 20 ps, while the protein and ions were restrained at a constant number-pressure-temperature (NpT) at 50 K and 1 atm by applying a force constant of 100 kcal·mol⁻¹·Å⁻¹. Then the system was gradually heated to 300 K via six 100 ps constant number-volume-temperature (NVT) MD simulations, still maintaining the restraint on the proteins. The restraints were gradually reduced to zero in the subsequent 200 ps equilibration simulation at NpT of 300 K and 1 atm. Lastly, 100 ns production simulations were carried out using Berendsen temperature and pressure coupling (24) without any restraint. The SHAKE constraints (25) were applied to all hydrogen heavy bonds to permit a dynamics time step of 2 fs. Electrostatic interactions were calculated by the particle-mesh Ewald method (PME) (26, 27) with grid spacing of 0.12 nm and interpolation of order four. Both of the direct space PME and Lennard-Jones cutoffs were set at 10 Å. The data for the MD simulations were collected every 2 ps.

2.4 Structural and dynamical analysis and electrostatic potential calculation.

All the analyses were performed using the equilibrated simulation trajectories by the PTRAJ program of AMBER. Secondary structural analysis, dynamical fluctuation analysis and electrostatic potential calculation were performed to better understand the change of KLHL3 caused by the mutations. Root mean square deviation (RMSD) of protein backbone atoms

was used to analyze the system's equilibration tendencies and the structural change of the Kelch domain and the acidic motif. We calculated the β strand occupancy for KLHL3 to evaluate secondary structural changes. The DSSP method was used to determine whether an amino acid residue belonged to an β strand (28). The occupancy of each residue in a β strand was determined on the basis of the percentage of time that the residue existed in the β strand over the simulation. The occupancy percentage for each system was calculated via dividing the sum of total occupancy percentage of each residue by the total residue number. Dynamical cross-correlation maps (DCCM) between residues were calculated to provide information for correlated motion of the pair residues and the degree of the correlation (29). MATLAB (30) was used to generate the cross-correlation plots.

Principal component analysis (PCA) was performed to characterize the collective motion of Kelch domain and WNK4 AM. In MD simulation, both local dynamical fluctuations and collective motions of residues occur simultaneously. PCA is a multivariate statistical method to separate the collective motions from the local dynamics by reducing high-dimensional motional data sets into a small subset composed of principal components that describes the collective motion (31). PCA describes protein motions by diagonalization of the covariance matrix C_{ij} ($3N \times 3N$ array) of the atomic positional deviations

$$C_{ij} = \langle (x_i - \langle x_i \rangle)(x_j - \langle x_j \rangle) \rangle$$

where x_i, x_j is a Cartesian coordinate of a residue, and the angle brackets represent the time averages over all simulation trajectories. After the matrix diagonalized, a set of eigenvectors and eigenvalues can be obtained. The eigenvectors indicate the directions of collective motion, and the corresponding eigenvalues indicate the amplitude of the motion. The major collective motion can be represented by large eigenvalues along the eigenvectors. In this study, simulation trajectories were projected on the area formed by the first two principal components.

The electrostatic potential for Kelch domain of KLHL3 was calculated by APBS (32) and mapped onto the molecular surface of Kelch domain using VMD (33). The average values and standard deviation for the variables of hydrogen bond and distance between Kelch domain of KLHL3 and AM of WNK4 were calculated. Significant differences for the studied variables were determined using the Student's t-test with 95% confidence (34).

3. RESULTS AND DISCUSSION

In this study, the systems containing one of the ten mutations were denoted as **Q309R**, **A340V**, **R384Q**, **L387P**, **S410L**, **S432N**, **S433N**, **A494T**, **R528H**, and **N529K**. The structure (PDB ID: 4CH9) reveals that half of the mutations (Q309R, S432N, S433N, R528H and N529K) are located at the AM-binding surface, whereas the other half (A340V, R384Q, L387P, S410, and A494T) are buried inside the Kelch domain (Figure S1). Results are presented in two groups based on whether the mutations are on the surface of Kelch domain or not, since differences were observed for the two groups during experiments and data analyses.

3.1 Assessing the effects of mutations in Kelch domain by western blot analyses.

Based on the current understanding that Kelch domain of KLHL3 binds with the AM of WNK4, PHAI1-causing mutations in the Kelch domain are expected to affect the Kelch-AM interaction. To verify this, we expressed a GST-acidic motif fusion construct (WNK4 AM) together with HA-tagged wild-type (WT) KLHL3 or KLHL3 with individual mutations at the Kelch domain in *Xenopus* oocytes. The protein abundance of KLHL3 and WNK4 AM constructs was assessed by western blot analyses (Figure 1). It appeared that the protein abundance of KLHL3 constructs with individual mutations were not different from that of WT KLHL3. Compared to WNK4 AM alone group, the co-expression of WT KLHL3 robustly reduced WNK4 AM abundance. All surface mutations in Kelch domain appeared to abolish the effect of KLHL3 on WNK4 AM. In contrast, only three buried mutations (i.e. R384Q, L387P, and S410L) impaired the ability of KLHL3 to reduce the protein level of WNK4 AM, whereas the A340V and A494T mutants behaved very similar to the WT KLHL3. These results are consistent with the observation by Ohta et al. that A340V and A494T mutations did not impair the effect of KLHL3 on WNK1 protein abundance as other mutations did (12). The western blot analyses demonstrate that the mutations of the Kelch domain act differently in the degradation of WNK4 AM. The abundance of WNK4 AM was determined by protein synthesis and degradation. KLHL3 is not known to regulate protein synthesis, thus, the change in WNK4 AM protein abundance in the presence of KLHL3 mutant reflects the change in protein degradation, which involves the binding between WNK4 AM and the mutated Kelch domain. To understand the mechanisms by which the mutations affect the Kelch-AM interaction, computational simulations were carried out and the results are presented below.

3.2 Structure and conformation of WNK4 AM were changed in the systems with a surface mutation.

Two simulation trajectories for WT Kelch domain in complex with WNK4 AM, denoted as **WT** and **WT'**, were performed to test the reproducibility of the simulation data. Results show that the two systems have similar values for root mean square deviation (RMSD), radius of gyration (Rg), root mean square fluctuation (RMSF), the distance between the Kelch domain of KLHL3 and AM of WNK4, and the secondary structure (see Figure S2, Figure S3, and the first row of Figure 2). These indicate that the simulations are reliable with good reproducibility, therefore only one trajectory was performed for each of the mutant systems.

First, the structural change of WNK4 AM was investigated by calculating the RMSDs relative to its initial structure (red lines in Figure 2). The RMSD values of AM in systems with a mutation buried inside the Kelch domain did not differ significantly from that in **WT**, except for **R384Q**. However, it was significantly increased in all systems with a mutation at the surface of the Kelch domain, indicating WNK4 AM underwent large structural changes in these systems. Thus, structural changes in AM correlates quite well with its protein abundance with respect to the effect of a co-expressed surface mutant or buried mutant R384Q, which may suggest a shared underlying cause, i.e., loss of the Kelch-AM binding in the presence of these mutations. To further investigate the structural change of the entire system, the RMSD for the Kelch-AM complex was calculated and shown in Figure S4. For WT systems, the RMSD values for the Kelch-AM complex were stable and were around 1 Å

during the simulations. However, the RMSD of the complex was increased in systems with a surface mutation (left panels in Figure S4), while it was not changed in systems with a buried mutation (right panels in Figure S4). This indicates that the mutations act differently to affect the Kelch-AM interaction, depending on whether they are on the surface or buried in the Kelch domain.

Principal components analysis (PCA) is usually used to identify the collective dynamics of residues that is related to conformational change (31). To further investigate the conformational change of WNK4 AM, PCA was performed and the last 40 ns simulation trajectories of AM were projected onto the first two PC (Figure 3). The AM in *WT* and *WT'* systems exhibited constrained motions in the restricted conformational space (regions in red dashed circles of Figure 3), indicating WNK4 AM is tightly bound to Kelch domain of KLHL3. However, the space explored by the AM varied in the mutant systems. For systems with an inside mutation, the projected areas were largely overlapped with those in *WT* and *WT'* systems, though the AM in *A340V* and *A494T* reached some regions which were not visited by *WT* and *WT'*. In contrast, despite the projected areas in *S432N* and *S433N* were largely overlapped with those in *WT* and *WT'*, the AM in the systems with a surface mutation explored larger areas compared to *WT* and *WT'*, indicating WNK4 AM has large conformational changes in these systems. Both the structural and conformational analyses suggest that the Kelch-AM binding is significantly disrupted by the surface mutations. This is consistent with the results shown in Figure 1 that the surface mutations drastically reduce the ability of KLHL3 in the degradation of WNK4 AM.

3.3 Electrostatic potential but not the structure of Kelch domain was altered in the systems with a surface mutation.

To test whether the Kelch domain of KLHL3, in addition to AM, undergoes structural changes when a mutation is present, we also calculated the RMSDs of the Kelch domain as a function of the simulation time (black lines in Figure 2). For all systems, the RMSD values remained stable at around 1 Å during the 100 ns simulation, indicating that none of the mutations led to drastic structural changes in the Kelch domain. Since the Kelch domain is characterized by its β -propeller structure, the β -strand occupancy was computed to examine whether the secondary structure of Kelch domain was changed (Figure S3). Compared to *WT* systems, none of the mutation systems showed significant change in the overall β -strand occupancy.

Our previous work (16) indicates that the surface of Kelch domain exhibits a positive potential to attract the AM which is rich in negatively charged residues. The Q309R, S432N, S433N, R528H and N529K mutations are located on the surface of Kelch domain, thus these mutations may alter the surface electrostatic potential for the AM binding site. To test whether this is the case, the surface electrostatic potential was calculated for Kelch domain in the presence of a surface mutation (Figure 4). The surface electrostatic potential for the AM binding site was mainly positive (blue areas in black dashed box) in the *WT* system. Compared to *WT* system, the electrostatic potential for the R528H mutation site (shown in green dotted circle) changed into negative values. Similar to the result of phosphorylation of KLHL3 at S433 (16), this negative potential may disfavor the binding of Kelch domain with

the negatively charged AM of WNK4. For the other four surface mutations, the electrostatic potential for the mutation sites (shown in green dotted circles of Figure 4) changed into positive values. These altered electrostatic potentials may drive WNK4 AM away from its originally binding site, and ultimately result in the disruption of the Kelch-AM interaction (discussed in part 3.4). For the mutations that are buried in Kelch domain, no change in the surface electrostatic potential were observed (data not shown).

3.4 Intermolecular hydrogen bond network was impaired in the systems with a surface mutation, allowing AM moving away from its original position

In the crystal structure, residues R339, R360 and R528 in the Kelch domain form intermolecular hydrogen bonds (H-bonds) with E559, E557 and D564 in WNK4 AM, respectively (13). These residues form an H-bond network which is important for stabilizing the Kelch-AM association. Our previous work (16) indicates that the disruption of the H-bond network is a key mechanism to destabilize the Kelch-AM association when serine 433 in the Kelch domain is phosphorylated. If a mutation in the Kelch domain influences any of these Kelch-AM H-bonds, their interaction may be altered. To assess this possibility, we computed the number of Kelch-AM H-bonds (Table 1). Four out of the five mutations in the surface of the Kelch domain, namely Q309R, S432N, R528H, and N529K, led to significant reduction in the number of the H-bonds. In contrast, none of the mutations buried inside the Kelch domain resulted in significant reduction in the H-bond number.

To define the position of AM relative to the Kelch domain containing individual mutations, we measured the distances between the three pairs of amino-acid residues that form the intermolecular Kelch-AM H-bonds (Figure 5). The three distances in *WT* and *WT'* systems were stable during the 100 ns simulation. We then used the *WT* systems to assess the dynamic changes in the distances between the three pairs of residues in the presence of a disease mutation (Figure 5). For the systems containing a surface mutation, all exhibited robust increases or decreases in at least two of the three distances measured. In contrast, the changes were modest (R384Q and L387P) or not detectable (A340V, S410L, and A494T) for the systems containing a buried mutation. Only the distance between E557 of WNK4 and R360 of KLHL3 was decreased slightly in the **R384Q** and **L387P** systems, and the distance between E559 of WNK4 and R339 of KLHL3 was modestly increased in the **L387P** system. To further investigate whether the mutations can impair the Kelch-AM interaction, the distance between the center of mass (COM) of the Kelch domain and that of AM was calculated and averaged in Table 1. Compared to *WT* system, the distance significantly increased in all the systems containing a surface mutation, indicating that AM shifted away from its original binding site in these mutation systems. No significant change in the distance between WNK4 AM and Kelch domain with a buried mutation was observed.

3.5 The mutations differentially affected the correlation motions of the systems.

The dynamical changes of the Kelch domain and AM were investigated by calculating the dynamical cross-correlation map (DCCM, Figure 6 and Figure S5). In DCCM, correlated motions between two residues are shown in red, whereas the anti-correlated motions between two residues are shown in blue. For *WT* systems, there were few correlated or anti-correlated motions within Kelch domain and between Kelch domain and AM of WNK4.

Significantly increased anti-correlated motions between Kelch domain and AM (regions in red dashed boxes of Figure 6 and Figure S5) were observed in surface mutation systems except for **S433N**, but not in systems with a buried mutation. Significant changes in anti-correlated motions between residues within Kelch domains (regions in black dashed boxes of Figure 6 and Figure S5) were observed in four out of the five systems with a buried mutation (i.e. **A340V**, **R384Q**, **L387P**, and **S410L**) and in system with **R528H** surface mutation. No remarkable changes in the correlated or anti-correlated motions were observed in **S433N** and **A494T** systems, which belong to systems with a surface and a buried mutation, respectively.

3.6 The mutations affected the binding between Kelch domain and WNK4 AM through different molecular mechanisms.

In the crystal structure, residues S432, S433, and R528 of KLHL3 are involved in forming intermolecular H-bonds with WNK4 AM, and residue R384 forms intramolecular H-bonds with S410 within Kelch domain of KLHL3. The mutations in the corresponding residues may impair the intermolecular or intramolecular H-bonds, and in turn result in decreased binding stability between Kelch domain and WNK4 AM or decreased structural stability of the Kelch domain. To assess the effect of the mutations on the corresponding H-bonds, the two mutation groups, i.e. surface and buried mutation groups, were further divided into two subgroups based on whether the mutation residue in the crystal structure is involved in H-bond formation.

In the first subgroup of surface mutations (subgroup **1A**), S432 and R528 form H-bonds with E562 and D564 of AM, respectively, in **WT** system, while S433 indirectly forms an H-bond with A563 of AM through a water molecule (left panels in Figure 7). These H-bonds stabilize the Kelch-AM binding. However, when the corresponding mutations were present, the H-bonds were disrupted (right panels in Figure 7). This may cause the increased dynamical flexibility of WNK4, and ultimately alters the Kelch-AM H-bond network (Figure 5 and Table 1).

In the second subgroup of surface mutations (subgroup **1B**), Q309 and N529 do not form an H-bond with WNK4 AM in **WT** system (left figures in Figure 8A and 8B). However, when the mutations were present, both mutated residues disrupted the key H-bond network (mentioned in part 3.4) by forming new intermolecular H-bonds with WNK4 AM (right figures in Figure 8A and 8B). For the Q309R mutation, the mutated residue R309 produced a shift toward positive electrostatic potential (Figure 4). This enables R309 to compete with R360 to bind with E557 of AM. As a result, new H-bonds formed between R309 and E557, instead of the H-bonds between R360 and E557 (Figure S6 and Figure 8A). Similar to Q309R mutation, N529K mutation also changed the electrostatic potential to a positive value (Figure 4). This let K529 compete with its neighboring residue R528 to bind with D564 of AM (Figure S7 and Figure 8B). Ultimately, the abnormal formation of the intermolecular H-bonds due to these two mutations attracted the AM moved away from the original binding site (Figure 8C).

For the systems with a buried mutation in the Kelch domain, changes in the electrostatic potential of the AM binding surface and in the intermolecular H-bond network were not

observed. However, the R384Q and S410L mutations altered intramolecular H-bonds to different extent within the Kelch domain. In the third subgroup (subgroup **2A**), the side chain of R384 formed H-bonds with both the main chain and hydroxyl oxygen atoms of S410 in **WT** system, which stabilize the tertiary structure of the Kelch domain. In the presence of R384Q mutation, however, these H-bonds were disrupted (Figure 9A). This was accompanied by the increased anti-correlated motions between residues within the Kelch domain (Figure S5). These changes likely caused the structural and dynamical instability of KLHL3, which in turn disrupt its interaction with WNK4. In contrast to R384Q mutation, an H-bond was still formed between side chain of R384 and the main chain oxygen of L410 in the presence of S410L mutation. To further understand how the S410L mutation affects KLHL3-WNK4 interaction, detailed analyses for the neighboring residues of S/L410 were performed. Secondary structure analysis showed that the neighboring residue V411 was in a β -strand and formed H-bonds with the main chain nitrogen and oxygen atoms of V424 in **WT**. However, in **S410L** system, the β -strand occupancy for V411 significantly decreased (99.81% in **WT** vs. 84.69% in **S410L** systems). In addition, RMSF values for the β -strands containing V411 and V424 increased in **S410L** system compared to those in **WT** (Figure 9B). The enhanced structural and dynamical flexibility of the neighboring residues caused by S410L mutation could reduce the ability of Kelch domain to bind with WNK4 AM.

The fourth subgroup (subgroup **2B**) includes the A340V, L387P and A494T mutations. For group **2B**, increased anti-correlated motions between residues within Kelch domain were observed in **A340V** and **L387P** systems but not in **A494T** system (Figure 6 and Figure S5). No significant changes were found in structure and electrostatic interactions for these systems. According to the western blot analyses (Figure 1), the L387P mutation did impair the ability of KLHL3 to reduce the level of WNK4 AM. In contrast, none of the A340V and A494T mutations does appear to affect the ability of KLHL3 in the degradation of WNK4 AM (Figure 1). These mutations might cause defects in the KLHL3 stability, which was not investigated in the current study although no significant change in protein abundance of KLHL3 mutants was observed.

Most results derived from the simulations of the interaction between Kelch domain of KLHL3 carrying disease-causing mutations and AM of WNK4 are highly consistent with the results obtained by western blot analyses (Figure 10). First of all, the alteration in H-bonds due to mutations in the Kelch domain revealed by the simulations correlates very well with the less degradation of WNK4 AM indicated by the higher protein levels from the western blot analysis. The mutations in S432, S433, and R528 (S432N, S433N, and R528H, Group 1A) disrupt their H-bonds with E562, A563, and D564 of WNK4 AM, respectively (Figure 7). In contrast, mutated residues R309 and K529 (Group 1B) form new H-bonds with E557 and D564 of WNK4 AM, respectively (Figure 8). This prevented the two WNK4 residues from forming H-bonds with R360 and R528 of KLHL3, respectively, as normally occur in the WT system. These alterations in systems with a surface mutation result in the disruption of the H-bond network critical to the Kelch-AM association (Figure 5 and Table 1). In addition, all the surface mutations directly alter the electrostatic potentials on the surface of Kelch domain where the AM of WNK4 docks (Figure 4). These are likely the main reasons that the AM of WNK4 moved away from its original binding site in the Kelch

domain containing a mutation at the binding surface (Table 1). The anti-correlated motions between Kelch domain and WNK4 AM (Figure 6 and Figure S5) were likely a reflection of reduced interaction between them.

In contrast to the disruption of intermolecular H-bond network by mutations at the surface of Kelch domain, intramolecular H-bonds appear to be affected in the Kelch domain of KLHL3 carrying R384Q and S410L mutations (Group 2A). These mutations directly disrupt the intramolecular H-bonds between R384 and S410 (Figure 9), which may lead to structural changes in the Kelch domain. The anti-correlated motions between residues within Kelch domain observed for systems with R384Q and S410L (Figure S5) likely reflect this change. These alterations in the Kelch domain likely lead to the reduced interaction with the AM of WNK4 observed by simulations and western blot analyses.

The Kelch domain carrying one of the two buried mutations in Group 2B, A340V or A494T, shows no signs of altered interactions with the AM of WNK4. By western blot analyses, the protein abundance of WNK4 AM in the presence of KLHL3 with either A494T or A340V mutation was not increased compared to the WT (Figure 1). Consistently, no sign of difference in the Kelch-AM interaction was identified between the A340V, A494T and WT systems by simulation (Figures 2, 3, and 5). The only observed change in these two mutants was an increase in the anti-correlated motion within the Kelch domain. Based on these observations, we conclude that the A494T and A340V mutations may not significantly alter the Kelch-AM interaction. It is possible that these mutations do not interfere with the Kelch-AM interaction. These mutations may affect the interaction of Kelch domain with other motifs of WNK4 or other potential KLHL3 substrates. It is also possible that these mutations alter the stability of KLHL3 itself, although our western blot analyses provide no evidence for this. Further biochemical and computational studies are needed to understand the pathogenesis of PHAII caused by these two mutations.

The leucine to proline mutation usually causes a significant change in protein function because the cyclic structure of the side chain of proline produces a conformational rigidity in protein structure. However, no major changes in secondary structure and dynamical fluctuation of residue 387 were found between **WT** and **L387P** systems (Figure S8). The western blot analyses suggest that the interaction between AM of WNK4 and Kelch domain containing L387P mutation is significantly decreased compared to WT system, however, no significant alteration was observed by simulations other than the modest changes in the distances between two pairs of residues that form intermolecular Kelch-AM H-bonds and the anti-correlated motion in the Kelch domain. These changes appear to be small relative to the reduction in the degradation of WNK4 AM observed by western blot analyses. Although the simulation results of the other nine mutations are very consistent with the actual WNK4 AM degradation assessed by western blot analyses, it is likely limitations of our simulation studies exist, which prevents us from obtaining more consistent results for the L387P mutation. We hope future studies will provide a better understanding of the mechanisms by which L387P mutation disrupts the Kelch-AM interaction.

Besides the mutations in the Kelch domain of KLHL3, PHAII-causing mutations in the AM of WNK4 may also disrupt the Kelch-AM interaction. The electrostatic interaction is

important for the binding between the AM of WNK4 and the Kelch domain of KLHL3 (upper left panel of Figure 4). The PHAII-causing mutations at the AM of WNK4 (e.g. E562K, D564A, and Q565E) are charge-changing substitutions that would alter the electrostatic interaction with the Kelch domain. Furthermore, residues E562 and D564 at the AM of WNK4 form H-bonds with S432 and R528 at the Kelch domain of KLHL3, respectively (Figure 7). The changes in electrostatic interaction and intermolecular H-bonds caused by these mutations likely disrupt the Kelch-AM association. In addition, since the AM sequence is conserved between WNK1 and WNK4, the disease-causing mutations in Kelch domain of KLHL3 likely affect the interaction with WNK1 and WNK4 in the same manner. Further studies are needed to clarify these issues.

4. CONCLUSION

Consistent results were obtained by western blot and MD simulation analyses on the Kelch-AM interaction for disease-causing mutations in the Kelch domain. Furthermore, MD simulation analyses provide insights into the difference in molecular mechanisms caused by mutations on the surface of versus buried inside the Kelch domain. The interaction is disrupted by the mutations on the surface of the Kelch domain due to the changes in the electrostatic potential and the disruption of the intermolecular H-bond network, both being important for stabilizing the Kelch-AM association. In contrast, MD simulations did not show significant difference in the Kelch-AM interaction in systems with the mutations buried inside the Kelch domain. However, the disruption of the intramolecular H-bonds within the Kelch domain likely resulted in anti-correlated motions between residues within the Kelch domain carrying the R384Q or S410L mutation, and this may ultimately affect the Kelch-AM interaction. For two of the buried mutations (A340V and A494T), no significant changes in the Kelch-AM interaction were observed by both western blot and simulation approaches, suggesting that these mutations may disrupt interactions not involving the acidic motif. Finally, for the buried L387P mutation, although not to the same degree, alterations in the Kelch-AM interaction were observed by MD simulation and western blot analyses; however, the underlying mechanism remains unclear. Taken together, the MD simulation data correlate well with the results from western blot analyses for nine out of the ten mutations. Mechanisms disrupted by mutations are elucidated for the five mutations on the surface of and two mutations (R384Q and S410L) buried inside the Kelch domain. Future studies are necessary to understand the mechanisms disrupted by the L387P mutation and to identify the targets of interaction disrupted by the A340V and A494T mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

KLHL3	kelch like family member 3
WNK4	with-no-lysine [K] kinase 4
PHAII	pseudohypoaldosteronism type II
MD	simulation molecular dynamics simulation
AM	acidic motif
RMSD	root mean square deviation
RMSF	root mean square fluctuation
H-bond	hydrogen bond
PCA	principal component analysis
DCCM	dynamical cross-correlation map
WT	wild type

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Figure 1. Western blot analyses for the degradation change of WNK4 acidic motif (GST-tagged) caused by binding with the KLHL3 mutants.

Totally, ten mutations on the Kelch domain of KLHL3 were investigated. The results for the system with surface mutations are shown in the left panels, while the results for the system with buried mutations are shown in right panels.

Figure 2. Time-dependent root mean square deviation (RMSD) for the Kelch domain of KLHL3 (in black) and the acidic motif (AM) of WNK4 (in red). The mutations on the surface of Kelch domain are shown in left panels, whereas those buried inside the Kelch domain are shown in right panels.

Figure 3. Principal component analysis (PCA) for the acidic motif (AM) of WNK4.

The mutations on the surface of Kelch domain are shown in left panels, whereas those buried inside the Kelch domain are shown in right panels. Projection of last 40 ns MD trajectories onto the first two PCA eigenvectors. The red dot circles represent the area that explored by AM in **WT** system.

Figure 4. Electrostatic potential for the Kelch domain carrying surface mutations. Positive potential is shown in blue, whereas negative potential is shown in red. The AM-binding site of KLHL3 is labeled in black box, and the mutation site is labeled in green circle. Electrostatic potential for the buried mutations are not shown since they are the same to the WT KLHL3.

Figure 5. Distance comparison between WT and mutant systems.

(A) Distance between R339 of KLHL3 and E559 of WNK4 are shown in black, that between R360 of KLHL3 and E557 of WNK4 are shown in red, and that between R528 of KLHL3 and D564 of WNK4 are shown in green. Residues on Kelch domain of KLHL3 are labeled in blue, and those on AM of WNK4 are labeled in black. (B) Changes of the three distances along the simulation time. The mutations on the surface of Kelch domain are shown in left panels, whereas those buried inside the Kelch domain are shown in right panels. For comparison, the ranges of the distances shaded in WT systems are also represented in the mutant systems.

Figure 6. Dynamical cross-correlation map (DCCM) for WT (top right) and mutant systems (bottom left).

The comparison between *WT* and *Q309R* is shown in left panel, whereas that between *WT* and *A340V* is shown in right panel. The red color shows the correlation between residues, whereas the blue color shows the anti-correlation between residues. Red dashed boxes represent correlation between Kelch domain and AM, whereas black dashed boxes represent correlation within Kelch domain.

Figure 7. Disruption of hydrogen bonds directly caused by S432N, R528H and S433N mutations. Residues on Kelch domain of KLHL3 are labeled in blue, and those on AM of WNK4 are labeled in red. The Kelch domain is shown in yellow and AM is in purple. The carbon, nitrogen, oxygen and hydrogen atoms for are colored in cyan, blue, red and white, respectively.

FIGURE 8. Interaction change in *Q309R* and *N529K* systems.

(A) Hydrogen bonds (H-bonds) were formed between E557 of AM and R360 of Kelch domain in *WT*; instead, H-bonds were formed between E557 of AM and R309 of Kelch domain in *Q309R*. (B) Hydrogen bonds (H-bonds) were formed between D564 of AM and R528 of Kelch domain in *WT*; instead, H-bonds were formed between D564 of AM and K529 of Kelch domain in *Q309R*. (C) Top view for the binding of Kelch domain (in yellow) with AM (in purple). Residues on Kelch domain of KLHL3 are labeled in blue, and residues corresponding to mutation are labeled in red and green, respectively. Purple arrows indicate the movement of AM.

Figure 9. Change of interaction and dynamics caused by R384Q and S410L mutations. (A) Compared to *WT* system, hydrogen bonds between residues 384 and 410 are impaired in *R384Q* system, but one hydrogen bond is kept in *S410L* system. (B) Comparison of RMSF between *WT* and *S410L* systems. The β strand containing residues 411–414 is represented in cyan shaded regions and that containing residues 420–424 is represented in grey shaded regions.

Figure 10. Summary of the observations (left) and mechanisms (right) for the interaction between the Kelch domain and acidic motif (AM).

The mutations in the Kelch domain are divided into two groups based on the major observations, including degree of degradation of WNK4 AM (mild, moderate, or severe) assessed by western blot analyses, the structural change of AM measured by RMSD values, and the change in the distance between the center of mass (COM) of Kelch domain and that of AM as well as the distances between three pairs of key residues in intermolecular H-bonds. The two groups are subsequently divided into four subgroups based on to what extent the mutation is involved in the change of H-bonds: S432N, S433N, and R528H directly disrupt the intermolecular H-bonds between the Kelch domain and AM; R384Q and S410L disrupt the intramolecular H-bonds within the Kelch domain; Q309R and N529K form new aberrant H-bonds with AM; A340V, L387P, and A494T cause no change in either intermolecular or intramolecular H-bonds. In addition, changes in electrostatic potential are identified in all systems with the surface mutations. Anti-correlated motions between the Kelch domain and AM are identified in most systems with the surface mutations, whereas anti-correlated motions within the Kelch domain are identified in most systems with the buried mutations.

Table 1.
Comparison of hydrogen bonds and distance between *WT* and mutation systems.

The number of hydrogen bonds (H-bonds) was calculated by summing the hydrogen bonds between R339, R360 and R528 of Kelch domain and E559, E557 and D564 of AM, respectively, during the last 40 ns simulation trajectories. The distance was calculated between center of mass (COM) of KLHL3 Kelch domain and that of WNK4 acidic motif. Compared to *WT* system, the H-bonds and the distances in the systems with surface mutations were significantly increased. * indicates the difference is significant.

	mutation on the surface of Kelch domain						mutation buried inside Kelch domain				
	WT	Q309R	S432N	S433N	R528H	N529K	R384Q	S410L	A340V	L387P	A494T
H-bonds	5.45 ± 1.55	2.06 ± 1.13*	2.82 ± 0.99*	3.20 ± 1.18	2.57 ± 1.14*	2.29 ± 0.94*	3.70 ± 1.30	5.19 ± 1.29	3.48 ± 1.26	6.29 ± 1.30	4.66 ± 1.25
Distance	18.57 ± 0.43	20.02 ± 0.74*	21.76 ± 0.44*	20.21 ± 0.37*	21.85 ± 0.71*	19.42 ± 0.31*	19.04 ± 0.32	18.64 ± 0.19	18.68 ± 0.29	18.56 ± 0.23	18.67 ± 0.19