The AIRE G228W mutation disturbs the interaction of AIRE with its partner molecule SIRT1

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Abstract

The autoimmune regulator (AIRE) protein works as a tetramer. It interacts with partner proteins to form the "AIRE complex" that pushes RNA Pol II stalling in the chromatin of medullary thymic epithelial cells. AIRE is the primary transcriptional controller in mTECs, promoting the expression of a large set of peripheral tissue antigen (PTA) genes in the thymus, which are implicated in the negative selection of auto aggressive thymocyte clones before they reach the periphery. Under normal conditions, the SIRT1 protein temporarily interacts with AIRE and deacetylates K residues of the AIRE SAND domain. Once the AIRE SAND domain is deacetylated, the binding with SIRT1 is undone, allowing the AIRE complex to proceed downstream. Considering that the *in silico* and *in vitro* binding of the (AIRE) SAND domain with SIRT1 provides a powerful model system for studying the dominant SAND G228W mutation mechanism, which causes the autoimmune APS-1 syndrome, here, we integrated computational molecular modeling, docking, dynamics between the whole SAND domain with SIRT1, and surface plasmon resonance using a peptide harboring the aminoacid residues 211 to 230 of SAND, to compare the structure and energetics of binding/release between AIRE G228 (wild-type) and W228 (mutant) SAND domain to SIRT1. We observed that the G228W mutation in the SAND domain negatively influences the AIRE-SIRT1 interaction. The troubled interaction might cause a disturbance in the binding of the AIRE SAND domain with the SIRT1 catalytic site, making it difficult for the AIRE complex to proceed downstream.

Key words AIRE, SAND domain, SIRT1 interaction, APS-1 mutation mechanism, molecular dynamics

Introduction

The human autoimmune regulator (*AIRE*) gene spans 14 exons at chromosome 21q22.3, encoding a 1635-nucleotide mRNA and a 545-amino acid protein (Aaltonen et al., 1994; Nagamine et al., 1997; Blechschmidt et al., 1999). Nearly 145 single-nucleotide mutations or large deletions have been observed along the *AIRE* gene (Wolff and Oftedal, 2019; Besnard et al., 2021; Human Gene Mutation Database available at https://www.hgmd.cf.ac.uk/ac/). Some mutations in the *AIRE* gene are related to autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy (APECED) (Finnish-German APECED Consortium 1997; Nagamine et al., 1997), also known as autoimmune polyglandular syndrome type 1 (APS-1) (OMIM entry # 240300). Most APS-1 mutations are inherited in an autosomal recessive manner, except for a group of monoallelic mutations clustered within the zinc finger domain of the first plant homeodomain (PHD1) (Oftedal et al., 2015; Wolff and Oftedal, 2019) and a G228W mutation at the SAND domain, which follows a dominant inheritance pattern (Cetani et al., 2001; Ilmarinen et al., 2005; Su et al., 2008).

The 56-kDa AIRE protein is a transcriptional regulator in medullary thymic epithelial cells (Giraud et al., 2012; Passos et al., 2018; Perniola, 2018; Giraud and Peterson, 2019), exhibiting five domains and four LXXLL motifs. The caspase activation and recruitment (CARD) domain is essential for AIRE dimerization. The nuclear localization signal (NLS) domain presents a nuclear localization signal that

mediates AIRE migration from the cytoplasm to the nucleus. The Sp100, AIRE-1, NucP41/75, and DEAF-1 (SAND) domains promote protein-protein interactions and chromatin binding, which play a role in the nuclear speckle localization of AIRE, and the PHD1 and PHD2 domains function as histone code readers, interacting and supporting the process of chromatin decondensation (Anderson et al., 2016; Kumar et al., 2001; Perniola et al., 2014; Giraud and Peterson, 2019). During its action in chromatin, AIRE interacts with several partner proteins forming the "AIRE complex," in which SIRT1 is one of the most critical deacetylating K residues in the SAND domain, permitting the proper function of AIRE (Incani et al., 2014; Abramson et al., 2010; Abramson and Husebye, 2016).

In addition to chromatin binding, the SAND domain may also have a regulatory role in the homomultimerization of AIRE (Ramsey et al., 2002a; Halonen et al., 2004). The G228W SAND domain mutation was the first dominant mutation associated with the aggressiveness of APS-1 (Cetani et al., 2001; Ilmarinen et al., 2005; Su et al., 2008; Wolff and Oftedal, 2019); however, the reason for its dominant character and the molecular mechanism of APS-1 pathogenesis are still elusive.

Considering that i) the deacetylation of SAND domain K residues mediated by the partner SIRT1 molecule is essential for AIRE function, promoting RNA Pol II stalling in chromatin (Vaquero et al., 2004; Giraud et al., 2012, Saare et al., 2012, Incani et al., 2014, Chuprin et al., 2015, Bheda et al., 2016), and ii) the G228W mutation occurs at a position located at six amino acid residues from one of the acetylated K residues (K222), which is further deacetylated by SIRT1 (Saare et al., 2012), in this study, we hypothesized that the proximity of the G228W mutation to the K222 residue might interfere with the interaction between the SAND domain and the SIRT1 protein.

To test this hypothesis, we integrated computational molecular modeling, docking, dynamics between the whole SAND domain with SIRT1, and surface plasmon resonance by using a peptide harboring the aminoacid residues 211 to 230 of SAND to compare the structure and energetics of binding/release between AIRE G228 (wild-type peptide) and W228 (mutant peptide) SAND domain to SIRT1. Overall, the results show that the G228W mutation leads to a weaker physical interaction between the peptide segment of the AIRE SAND domain surrounding the mutant residue and the SIRT1 catalytic site than the equivalent wild-type segment.

Materials and Methods

Protein structure homology modeling

We used the SWISS-MODEL workspace for protein modeling (http://swissmodel.expasy.org/workspace). The amino acid sequence in the FASTA format of the SIRT1 target protein was used as input. The SWISS-MODEL algorithms performed BLASTp and provided the best homology models based on global model quality estimation (GMQE) and qualitative model energy analysis (QMEAN) statistical parameters. The GMQE provided the quality by combining the target-template alignment properties, estimating a range between 0 and 1. The scoring function of QMEAN consisted of a linear combination of structural descriptors, which is related to the high-resolution X-ray crystal structure, estimating a *Z*-score scheme.

Molecular docking

Molecular docking analysis of the modeled AIRE SAND domain, wild-type or G228W mutant AIRE SAND domain, and the SIRT1 protein (PDB code 4I5I) was performed to study the stability of the protein-protein interaction. The ClusPro 2.0 protein-protein docking server (Alekseenko et al., 2020) (available at https://cluspro.org/) was used. In the ClusPro server, the rigid body docking phase uses the PIPER docking program (https://www.schrodinger.com/products/piper), which relies on the fast Fourier transform (FFT) correlation approach. PIPER represents the interaction energy between two proteins using the formula $E = w1Erep+w2Eattr+w3Eelec+w4EDARS$, where $E\ rep$ and $E\ attr$ denote the attractive and repulsive contributions to the van der Waals interaction energy, and $E\ elec$ represents the electrostatic energy. $EDARS$ is a pairwise structure-based potential; it primarily means desolvation contributions, i.e., free energy change by removing the water molecules from the interface. The coefficients $w\ 1$, $w\ 2$, $w\ 3$, and $w\ 4$ define the molecular weights of the corresponding amino acid residues.

Systems setup and molecular dynamics simulations

As mentioned above, the three-dimensional (3D) structures of the complexes formed with AIRE wild-type or with AIRE mutant peptide and SIRT1 generated in the docking section were used as initial configurations for the molecular dynamics simulations. In this work, calculations were performed to verify the stability and affinity of the complexes.

To mimic the interaction between the two peptides and SIRT1 protein, the K222 residue was acetylated using the parameters given in the AMBER Molecular Dynamics Package (https://ambermd.org/) for this residue. ACK and the connectivity library file for K222 residue were generated by tleap.

The two systems were solvated using the TIP3P water model and neutralized with four Na+ ions in the tleap tool implemented in AMBERTOOLS21 (https://ambermd.org/AmberTools.php). The whole system was truncated in a box with 15 Å between the edges of the periodic box and the molecules analyzed. An 8 Å cutoff particle mesh Ewald (PME) method was used to treat the long-range electrostatic interactions. AMBER molecular dynamics software and the ff19SB force field were used to prepare the systems and run the simulations.

Before running, the complexes were subjected to minimization in two stages using the steepest descent method followed by a conjugate gradient. In the first stage, the elements, peptides, and SIRT1 were fixed, and the position of the water molecules was minimized. After that, the whole system was minimized. A restrained thermalization phase with the microcanonical ensemble (NVE) was performed, increasing the temperature by 0.1 K until it reached 300 K using the Langevin thermostat and SHAKE algorithm (available at https://ambermd.org/) to constrain hydrogen bonds.

The production dynamics were carried out at constant pressure (NPT ensemble) with a time step of 2 fs. The total simulation time for each complex was 2700 ns, and

frames were saved every 20 ps. The analyses were performed in the last 2650 ns after the systems were equilibrated. All calculations shown in the current work were performed using AMBERTOOLS21 scripts. The visualization programs used to create the figures of the molecules were VMD for LINUXAMD64, version 1.9.3, and PYMOL (available at https://pymol.org/2/).

Surface plasmon resonance

The surface plasmon resonance (SPR) experiments were performed on Biacore Model T200 equipment (GE Healthcare Biosciences, Uppsala, Sweden). For the immobilization of human SIRT1 (catalog number S8846, Sigma–Aldrich, Saint Louis, MO), a CM5 sensor chip S series (GE Healthcare Biosciences), which has carboxylic groups available for covalent coupling, was used. All solutions used were purchased from the equipment supplier (GE Healthcare Biosciences). The procedures described here were performed automatically with the Biacore instrument using pre-established programs for binding amines present in peptides or proteins.

Briefly, the carboxylic groups present on the CM5 sensor chip surface were activated with EDC + NHS, forming the NHS ester reactive intermediate, which, after washing with HBS-N, readily reacted with amines dissolved in the immobilization solution. The human SIRT1 ligand was dissolved in 10 mM sodium acetate pH 4.0 plus 20 µM NAD+ (catalog number N1636, beta-nicotinamide adenine dinucleotide hydrate, Sigma–Aldrich), which was immobilized onto the Biacore sensor chip according to the manufacturer´s instructions.

To detect interactions between SIRT1 and AIRE WT or AIRE G228W mutant SAND domain peptides, different concentrations of peptides were injected onto the CM5 chip with immobilized SIRT1 protein. Peptide samples were injected sequentially from lowest to highest concentrations (20, 100, and 200 µM) with a contact time of 300 s, a 20 µl/min flow, and a dissociation time of 1200 s. The surface was regenerated between samples by injecting 2.0 M glycine pH 2.0 (30 s, 30 µl/min) followed by injection of 2.0 M NaCl (30 s, 30 µl/min). The stabilization period between injections after regeneration was 200 s.

The respective sequences of the twenty-amino acid peptide residues (Synbio Technologies, Monmouth Junction, NJ, USA) comprising positions 211 to 230 of the SAND domain of the human AIRE protein are as follows: from the C to N-terminus, AIRE wild-type (IQQVFESGGSK<u>K</u>CIQVGGEF) and AIRE G228W mutant (IQQVFESGGSK<u>K</u>CIQVGWEF). Depending on the experiment, the K residue underlined was acetylated or not acetylated. The AIRE wild-type or AIRE G228W mutation data are available through GenBank NCBI (https://www.ncbi.nlm.nih.gov/) accession numbers (NM_000383.4): c.682G>T G [GGG] > W [TGG], coding sequence variant autoimmune regulator (NP_000374.1): p. Gly228TrpG (Gly) > W missense variant, DbSNP (https://www.ncbi.nlm.nih.gov/snp/rs121434257) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/variation/3313/).

Kinetic data were analyzed with Anabel Analysis of Binding Events software (Krämer et al., 2019), available at (www.skscience.org/anabel). Data obtained with the different concentrations of AIRE WT or mutant peptides were combined in the same graph.

Results

Homology molecular modeling of the AIRE *SAND domain*

To obtain a separate 3D human AIRE SAND domain structure, we used the SwissModel tool to model proteins through homology. We used the SAND domain sequence of the human SP100 protein (PDB code 1h5p) for the modeling, which features 39.62% sequence identity to the human AIRE SAND domain. The SP100 protein SAND domain conserves the SKKCIQ amino acid sequence in the AIRE SAND domain, which corresponds to the accurate interaction site between SAND and the SIRT1 protein. Thus, it was possible to obtain a 3D structure of the wild-type AIRE SAND domain and the AIRE SAND domain carrying the G228W mutation. The G228W substitution did not cause misfolding in the mutant SAND domain structure (Figure 1).

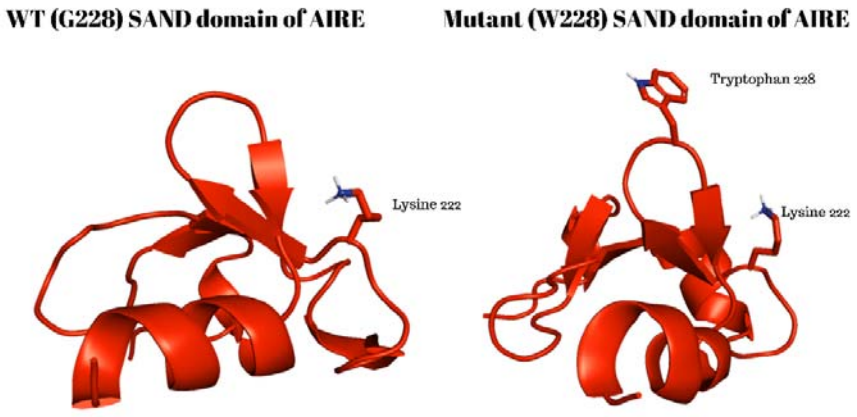

Figure 1. Molecular modeling of the G228 wild-type (left) or mutant W228 AIRE SAND domain (right) as obtained through homology with the human SP100 SAND domain.

Blind molecular docking shows that the SAND W228 mutant residue was targeted to the SIRT1 *catalytic residue*

Through blind molecular docking, it was possible to observe an affinity between the AIRE SAND domain WT G228 or W228 mutant domain and the catalytic region/site (H363 residue) of the SIRT1 protein, even without any previous targeting or chemical modification of the K222 residue, which is a target of the SIRT1 protein (Figure 2).

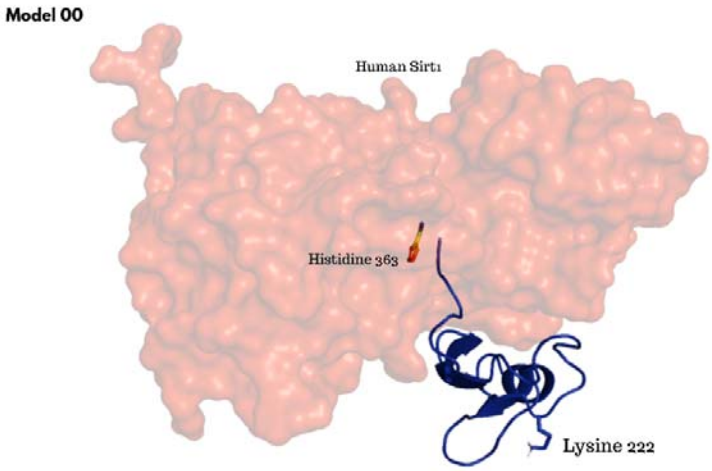

Figure 2. Docking output of the wild-type AIRE SAND domain showing that this domain interacts with the catalytic region of the SIRT1 protein.

Comparing the structural conformations, we observed that the SAND domain mobilizes the W228 mutant residue that interacts with the SIRT1 H363 catalytic residue in 7 out of 10 docking analyses (Figure 3).

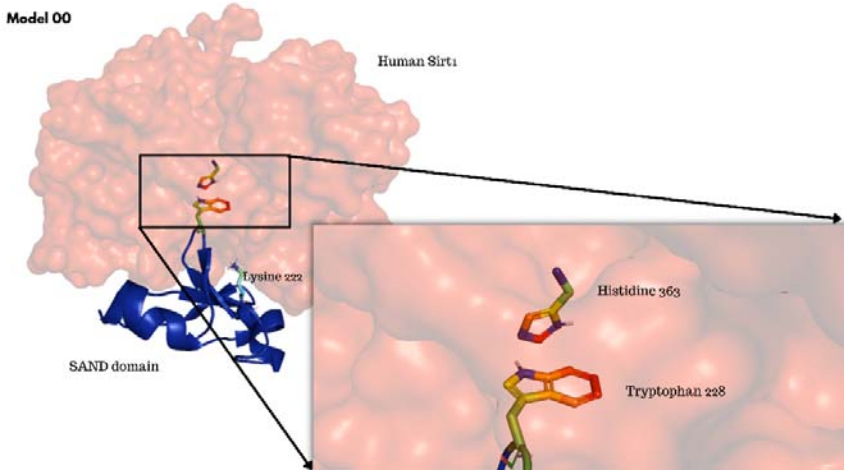

Figure 3. Docking output of the mutant W228 AIRE SAND domain showing the W228 residue from the AIRE side interacting with the H363 catalytic residue from the SIRT1 side. Blind docking indicated the targeting of mutant residue W228 to catalytic residue H363 of SIRT1.

Molecular dynamics

The molecular dynamics results show that the complex formed between the WT SAND domain and SIRT1 is slightly less stable over the simulation time than the complex formed between the SAND G228W mutant. Nevertheless, the G228W mutant domain complex remained stable without significant jumps in the RMSD (Figure 4).

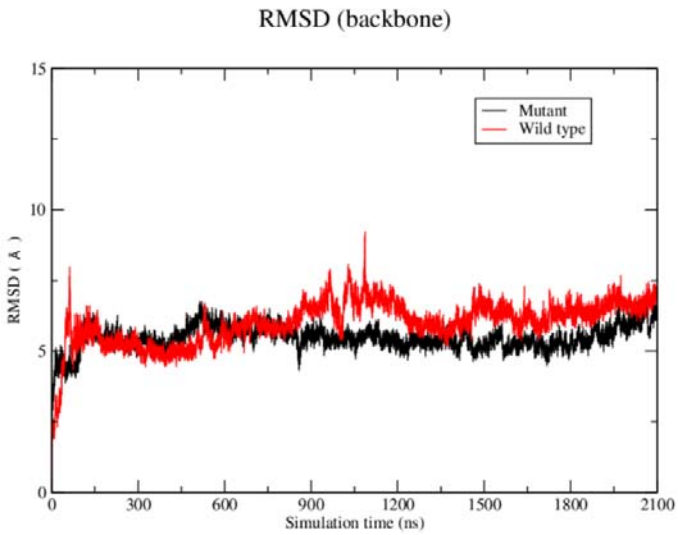

Figure 4. Molecular dynamics of the wild-type (red) or mutant W228 (black) AIRE SAND domain with the SIRT1 protein.

The analysis of structures from molecular dynamics showed that even having inserted an acetyl radical at K222 residue in the WT or mutant domains, there was no targeting to the catalytic residue H363 of SIRT1 (Figures 5 and 6).

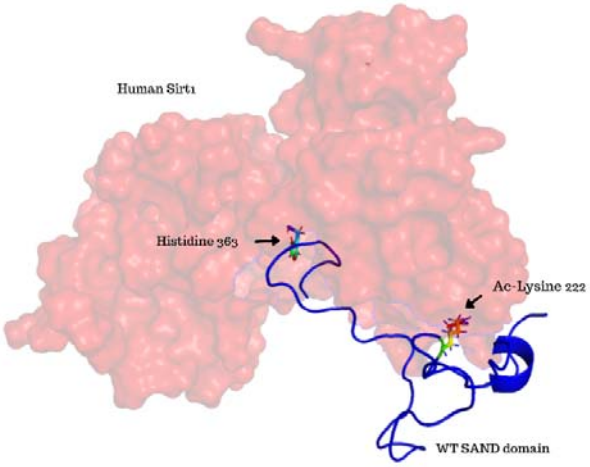

Figure 5. Molecular dynamics structure formed between the wild-type AIRE SAND domain and SIRT1 protein. Note that the acetylated K222 residue from the AIRE side is not directed to the SIRT1 H363 catalytic residue during the analyzed dynamics time (1.0 microsecond).

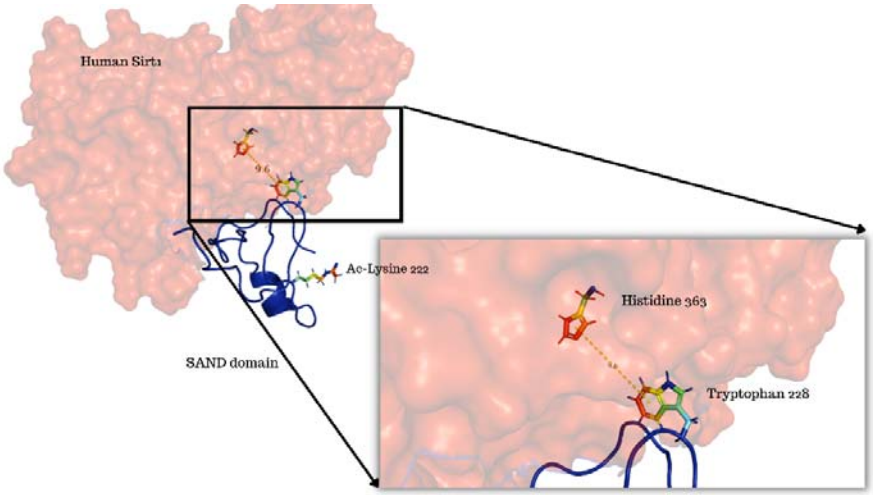

Figure 6. Molecular dynamics structure formed between the mutant W228 AIRE SAND domain and SIRT1 protein. Note that at 1.0 microseconds of dynamics, the mutant residue W228, seen from the side of AIRE, remained close to the catalytic H363 residue of SIRT1.

Surface plasmon resonance indicates that the SAND G228W mutation modifies the interaction kinetics of AIRE with SIRT1

Surface plasmon resonance (SPR) results indicated a differential association and dissociation dynamics between the wild-type SAND G228 or mutant SAND W228 peptides and the SIRT1 protein. The interaction kinetics showed that the wild-type SAND G228 peptide has a more significant association capacity as observed at a concentration range of 100 to 200 µM. The interaction between the wild-type G228 SAND peptide and SIRT1 was almost wholly undone after 1200 seconds of measurement. Conversely, the mutant W228 SAND peptide was comparatively less able to associate with SIRT1 (Figure 7). These results are experimentally convergent to the computational modeling and molecular dynamics showing that the G228W mutation interferes with the SAND-SIRT1 interaction.

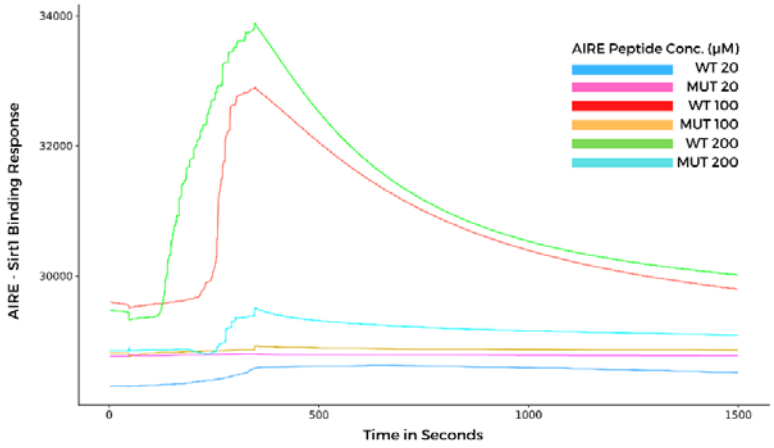

Figure 7. Surface plasmon resonance sensorgrams for binding of the AIRE SAND domain with SIRT1 protein immobilized onto a sensor chip. The peptides were assayed at various concentrations (20, 100 and 200 µM) with a contact time of 300 seconds, a 20 µl/min flow and a dissociation time of 1200 seconds.

Discussion

We reported that the dominant G228W mutation observed at the SAND domain of the human AIRE protein found in APS-1 patients interferes with the interaction with the deacetylase SIRT1 compared to the AIRE wild-type protein. Because of the interference of the W228 mutant residue, the kinetics of the association and dissociation processes differ compared to the wild-type, which might explain the nuclear accumulation of the AIRE complex, as observed in AIRE mutant cells (Ilmarinen et al., 2005). The binding/dissociation kinetics between AIRE-SIRT1 is crucial, and any disturbance in these processes may destabilize the catalytic action of SIRT1 for AIRE protein deacetylation. Thus, the model system employed in this work contributes to improving the understanding of the molecular mechanism of the G228W mutation.

The study of the molecular mechanisms of primary immunodeficiencies associated with the development of autoimmune disorders has contributed to understanding the mechanisms of tolerance loss to self-antigens. Although recessive AIRE mutations at the PHD1 domain may occur at a ratio of 1:1000 in the general population (Anderson et al. 2014; Su et al. 2016), AIRE SAND G228W is the unique dominant mutation associated with APS-1 (Cetani et al., 2001; Ilmarinen et al., 2005; Wolff and Oftedal, 2019). In the context of dominant inheritance, the central question is how these mutations influence the function of AIRE at the molecular level. Halonen et al. (2004) and Ilmarinen et al. (2005) observed that the G228W mutation quantitatively alters the nuclear location of the AIRE protein, impairing its transactivating property. Furthermore, Anderson and Su (2016) demonstrated that this mutation interferes with the localization of the AIRE protein, reducing the transcription of peripheral tissue antigens (PTAs).

This study evaluated whether the G228W mutation interferes with binding with SIRT1, an essential partner protein of the AIRE complex. We accessed the 3D structure for the AIRE SAND domain by homology modeling to the human SP100 protein SAND domain, which is available in a public database. We observed that the G228W replacement, which occurs in a loop region, did not result in misfolding of the AIRE SAND 3D structure, which could itself alter protein functionality. The wild-type or mutant AIRE SAND domain did not differ in their respective 3D structures, as performed by homology. This may be due to the segment's characteristics in which the G228W mutation is present, taking the form of a flexible loop region (SAND amino acid residues 226-228). Therefore, the G228W mutation must involve another mechanism. Next, we asked whether the mutation could interfere with AIRE binding to one of its partner proteins. To initiate the downstream cascade of interactions, AIRE needs to be deacetylated until it pushes RNA Pol II stalling in the chromatin. As SIRT-1 is the only deacetylase that integrates the AIRE complex, we next evaluated the binding of the AIRE SAND domain to SIRT1.

To obtain an interaction model between the AIRE SAND domain (wild-type or G228W mutant) and SIRT1 and assess the affinity of the mutant SAND domain, we used the ClusPro 2.0 blind molecular docking tool. For this purpose, the structures of the wild-type SAND domain, the G228W mutant output from homology modeling, and the structure of the SIRT1 catalytic domain (PDB code 4I5I) were used. We observed that the SAND domain mobilizes the W228 mutant residue that interacts with the SIRT1 H363 catalytic residue, interfering with the interaction kinetics between these proteins, followed by surface plasmon resonance measurements. Due to the SAND G228W mutation´s interference, the AIRE protein could bind weakly to SIRT1, as

observed in this study, but become more tightly bound to chromatin. In addition, to signify a nonphysiological situation, this finding might explain the accumulation of the AIRE complex in the nucleus of mutant cells, as previously reported (Halonen et al. 2004; Ilmarinen et al. 2005).

The AIRE protein plays a role in the cell nucleus as a tetramer (Abramson et al., 2010; Incani et al., 2014), in which the SAND domain of each subunit has K residues SIRT1 deacetylates. SIRT1 exerts its enzymatic activity, which depends on the amino acid residues near the acetylated K residues in the AIRE SAND domain (Garske et al., 2006). Accordingly, we hypothesized that the G228W mutation close to acetylated K residues could interfere with the SIRT1 interaction since it is an enzyme necessary to convert the acetylated to the nonacetylated and functional form of AIRE.

Several lines of evidence stress the importance of K deacetylation in the proper interaction of AIRE with SIRT1: i) functional analyses of the G228W AIRE SAND mutation show that the subunit-containing acetylated K residue could form a hybrid tetramer unable to promote AIRE transactivation activity (Halonen et al. 2004; Ilmarinen et al. 2005), ii) AIRE mutations mimicking acetylated K243 or K253 residues in the SAND domain also reduced the transactivation activity and accumulated as nuclear bodies, whereas mutations mimicking nonacetylated K residue were functionally similar to wild-type AIRE (Saare et al. 2012), and iii) the AIRE SAND R247C mutation retains the ability to form complexes with wild-type AIRE and localize into the nucleus; however, it reduces the activation of downstream transcription through dominant-negative inhibition (Abbott et al. 2018). Notably, the R247C mutation occurs close to other K residues (K243 and K253) in the SAND domain that SIRT1 can deacetylate, also harming the transactivation function of AIRE.

The molecular dynamics results with the AIRE SAND domain (wild-type or G228W mutant) showed that even the acetylated K222 residue at 1 µs of simulation did not allow the wild-type domain to form a stable structure, in which the K222 residue would interact with the H363 residue from the catalytic site of SIRT1. On the other hand, the G228W mutant domain exhibited a stable interaction with the SIRT1 catalytic site at the H363 residue. To verify the stability of both simulations and the molecular interaction, the atomic position of the backbone atoms relative to the reference structure was calculated using root mean square deviation (RMSD). This approach confirmed the hypothesis raised in this study, in which we consider that the AIRE G228W mutation interferes with SIRT1 binding.

To validate the *in silico* studies referring to the different interactions of the G228W SAND mutant with the SIRT1 protein, we performed an *in vitro* study to evaluate this interaction using surface plasmon resonance (SPR). Sensor-based SPR is a label-free, sensitive, precise, and *in vitro* tool used for determining binding kinetics between biological molecules, including antigen-antibody or protein-protein interactions, among other molecular interactions (Nguyen et al., 2015; Wang et al., 2019; Soltermann et al., 2021; Ribeiro et al., 2021).

This approach allowed us to evaluate the association/dissociation kinetics of the AIRE SAND domain peptides, wild-type, or those encompassing the G228W mutation, which interacts with the SIRT1 protein. We were able to observe that both peptide types can bind to SIRT1. In contrast, the wild-type SAND domain peptide binds faster and

dissociates longer, and the binding kinetics of the G228W mutant peptide feature a weak association. The results provide a precise molecular basis for clarifying how the mutant G228W AIRE protein loses its function.

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Contributions

JCS: raised hypothesis, discussed the analysis tools, performed molecular docking, analyzed data and interpreted the results.
MD: discussed the analysis tools, performed molecular dynamics, analyzed data and interpreted the results.
APM: performed protein binding kinetics, and interpreted the results.
VMF: performed protein binding kinetics and interpreted the results.
RB: performed molecular dynamics, analyzed data.
EAD: raised hypothesis, interpreted the results and wrote the manuscript.
GAP: conceived the study, raised hypothesis, organized the study pipeline, interpreted the results and wrote the manuscript.

Competing interests

The authors declare no competing interests.