NMR Structure of the First PHD Finger of Autoimmune Regulator Protein (AIRE1)

INSIGHTS INTO AUTOIMMUNE POLYENDOCRINOPATHY-CANDIDIASIS-ECTODERMAL DYSTROPHY (APECED) DISEASE*\[S\]

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Mutations in the autoimmune regulator protein AIRE1 cause a monogenic autosomal recessively inherited disease: autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). AIRE1 is a multidomain protein that harbors two plant homeodomain (PHD)-type zinc fingers. The first PHD finger of AIRE1 is a mutational hot spot, to which several pathological point mutations have been mapped. Using heteronuclear NMR spectroscopy, we determined the solution structure of the first PHD finger of AIRE1 (AIRE1-PHD1), and characterized the peptide backbone mobility of the domain. We performed a conformational analysis of pathological AIRE1-PHD1 mutants that allowed us to rationalize the structural impact of APECED-causing mutations and to identify an interaction site with putative protein ligands of the AIRE1-PHD1 domain. The structure unequivocally exhibits the canonical PHD finger fold, with a highly conserved tryptophan buried inside the structure. The PHD finger is stabilized by two zinc ions coordinated in an interleaved (cross-brace) scheme. This zinc coordination resembles RING finger domains, which can function as E3 ligases in the ubiquitination pathway. Based on this fold similarity, it has been suggested that PHD fingers might also function as E3 ligases, although this hypothesis is controversial. At variance to a previous report, we could not find any evidence that AIRE1-PHD1 has an intrinsic E3 ubiquitin ligase activity, nor detect any direct interaction between AIRE1-PHD1 and its putative cognate E2. Consistently, we show that the AIRE1-PHD1 structure is clearly distinct from the RING finger fold. Our results point to a function of the AIRE1-PHD1 domain in protein-protein interactions, which is impaired in some APECED mutations.

Mutations in the autoimmune regulator gene 1 (AIRE)\[1\] give rise to autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)\[1, 2\], a rare monogenic disease with autosomal recessive inheritance. The APECED disease results from poor tolerance to self-antigens, leading to destructive autoimmune disorders of the endocrine organs, chronic mucocutanous candidiasis, hypoparathyroidism, and adrenocortical failures (OMIM 240300). Over 50 pathological mutations, including truncations and point mutations, have been identified throughout the coding region of the AIRE1 gene\[3\]. AIRE1 encodes the multidomain AIRE1 protein (545 amino acids) of largely unknown function. AIRE1 is predominantly localized in the nuclear matrix\[4\] and is most highly expressed in immunologically relevant tissues such as the spleen and thymus. AIRE1 has transcriptional activating properties\[5\] regulating the thymic transcription of peripheral tissue self-antigens\[6, 7\]. The protein harbors an N-terminal homogeneously staining region\[8\], a nuclear localization signal, four LXXLL motifs, a SAND domain likely to mediate DNA binding\[8–10\] and two plant homeodomain (PHD)-type zinc fingers\[11\]. Both of the PHD fingers are mutational hot spots for AIRE1, they contain the sites of several pathological point mutations and are absent in several APECED-causing truncation mutants\[12\], thus suggesting a crucial role for the domains.

The PHD finger is a domain of ~60 amino acids found in over 400 eukaryotic proteins, many of which are involved in the regulation of gene expression and in the maintenance of chromatin structure\[11\]. Recent studies have suggested a role for PHD fingers as nucleosome interaction determinants\[13, 14\]. However the function of the PHD finger is still elusive and controversial, as a variety of functions have been suggested, including phosphoinositide binding\[15\] and E3 ubiquitin ligase activity\[16, 17\].

To gain more insight into the structure-function relationship of the AIRE1 PHD fingers and their role in the pathogenesis of APECED, we have determined the solution structure of the first PHD finger of AIRE1 (hereafter termed AIRE1-PHD1). The comparison with previously determined PHD structures shows that AIRE1-PHD1 presents the typical ββα topology.

\[1\] The abbreviations used are: AIRE1, autoimmune regulator 1; RMSD, root mean squared deviation; PDB, Protein Data Bank; GST, glutathione S-transferase; DTT, dithiothreitol; PHD, plant homeodomain; NMR, nuclear magnetic resonance; Ub, ubiquitin-conjugating enzyme (H suffix indicates human enzyme); NOE, Nuclear Overhauser effect; NMR, Biomolecular NMR Laboratory; DTT, dithiothreitol; BHV, Kapo- si’s sarcoma-associated Herpes virus; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; MDM2, mouse double minute 2 protein.

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and binds two zinc ions in a cross-brace coordination scheme. The structure, together with the conformational analysis of APECED mutants, allows a rationale for the structural impact of pathological point mutations. Finally, we sought possible functions for AIRE1-PHD1. Based on the fold similarity with RING fingers, PHD fingers have been proposed to function as E3 ligases in the ubiquitin-mediated proteosomal degradation pathway (16, 18), although this hypothesis is hotly debated in the literature (19, 20). At variance to a recent study (21), we could not observe an E3 ubiquitin ligase activity of the AIRE1-PHD1 domain.

EXPERIMENTAL PROCEDURES

Sample Preparation for NMR and Binding Assays—All expression constructs of human AIRE1 (GenBank™ NM_006689) were prepared in modified pET-22d vectors (Novagen Inc., Madison, WI) by PCR cloning from an AIRE1 template kindly provided by Dr. P. Peterson (University of Tartu, Estonia). The AIRE1-PHD1 construct used for structure determination spans wild-type residues Glu293-Glu354. The full-length AIRE1 and AIRE1-PHD1 were also cloned into modified pET-20M and pET-24d vectors enabling expression as thioredoxin- and GST fusion proteins, respectively. However the yields of full-length AIRE1 constructs were extremely low and insufficient to test their activity accurately. Site-directed mutations were made by standard overlap extension methods. The DNA sequences of all constructs were verified in-house and the molecular weights were verified by mass spectrometry (MALDI). The modified pET-24d vectors express proteins with N-terminal His6 tags, removable by cleavage with TEV (tobacco etch virus) protease, enabling use of non-tagged proteins in NMR studies.

The protein purification strategy was as described previously (9), except that here 50 μM ZnCl2 was added to the growth media and purification buffers. The purified sample was exchanged into 50 mM sodium phosphate pH 6.5, 0.15 M NaCl, 5 mM DTT, 50 μM ZnCl2, 0.02% NaN3, 10% (v/v) D2O and kept under argon gas for NMR experiments, or 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 50 μM ZnCl2, 0.02% NaN3, 15N relaxation rates were measured using standard two-dimensional methods (30). The relaxation delays were applied in an interleaved manner. The T1 and T2 decay curves were sampled at 14 different time points (20–1600 ms, 14.4–244.8 ms, respectively). The heteronuclear 1H-15N NOEs as well as longitudinal and transversal 15N relaxation rates were measured using standard two-dimensional methods (30). The relaxation delays were applied in an interleaved manner. The T1 and T2 decay curves were sampled at 14 different time points (20–1600 ms, 14.4–244.8 ms, respectively). The heteronuclear NOEs were calculated twice in an interleaved fashion with and without (reference spectrum) proton saturation during proton recovery delay. The relaxation experiments were analyzed with the program Nmrview.5.03 (31). Relaxation rates were obtained from non-linear least-square fits using software developed in-house.

For NMR titrations the 15N-labeled AIRE1-PHD1 domain and the tetratricopeptide repeat was determined performing a two-dimensional HMQC experiment optimized to detect J-couplings in histidine side chains (28). The 1H, 15N and 13C chemical shifts of the backbone resonances were obtained from sensitivity-enhanced three-dimensional HNCA, CBCA(CO)NH, and CBCANH experiments. The side chain signals were assigned from three-dimensional HCCONH-TOSCY, CCONH-TOSCY, and HCCH-TOSCY experiments (27). The tautomeric state of the histidine ring was determined performing a two-dimensional 1H-15N HMBC experiment optimized to detect J-couplings in histidine side chains (28).

The non-proton distances were calculated using the coordi- 15N- and 13C/15N-labeled glucose. NMR experiments were performed with a protein concentration of 0.5–1.0 mM.

Structure Calculations—Structures were calculated using ARIA (1.2) (32) in combination with CNS using the experimentally derived restraints (970 NOEs, 66 dihedral angle restraints, 44 residual dipolar couplings, and 6 hydrogen bonds). All NOE restraints were assigned manually and calibrated by ARIA. However, the automated assignment of ARIA was not satisfactory. A total of eight iterations (20 structures in the first 6 iterations) were performed: 100 structures were computed in the last two iterations. The ARIA default water refinement was performed on the 20 best structures of the final iteration.

Initial structures were calculated without zinc ion restraints to verify the position and the geometry of the metal ion ligands, so that the residues involved in zinc binding could be identified in an unbiased manner. Several NOEs were observed between metal coordinating residues, clearly revealing the tetrahedral coordination of the ligands around the coordinated zinc. After the metal ligands were unequivocally identified, in the final refinement calculation, distance restraints for the Zn-S, and Zn-N bonds were added (2.3 ± 0.5, 2.1 ± 0.5 Å, respectively). The presence of these additional restraints resulted in only a modest shift in the backbone of the AIRE1-PHD1 structure, leading to only a small increase of the RMSD for the backbone atoms (0.65 ± 0.11 and 0.72 ± 0.15 Å, with and without zinc restraints respectively). Importantly, in these final calculations none of the pre- viously determined NOE restraints were violated thus confirming the correct geometry of the ligands.

Structural quality was assessed using PROCHECK-NMR (33). Molecular images were generated using MOLMOL (34). Structure similarity searches were performed using the DALI server (www.ebi.ac.uk/ dali/index.html) (35). The structure of AIRE1-PHD1 (residues Glu123 to Glu485) was generated by comparative modeling (36) using the coordinates of AIRE1-PHD1 and the Protein Data Bank (PDB) entries 1F62, 1MM2, and 1FP0 as templates.

The family of the 20 lowest energy structures (PDB accession code 11506).
IXWH has been deposited in the PDB. Chemical shift and restraints that were used in the structure calculations have been deposited in BioMagResBank (accession code 6374).

RESULTS

Structure Determination—The solution structure of a construct encompassing residues Gin329–Glu354 (AIRE1-PHD1) containing the first PHD finger of AIRE1 was determined by multidimensional heteronuclear NMR spectroscopy (Fig. 1). The recombinant protein behaves as a monomer in solution, as assessed by the rotational correlation time (τc ~ 4 ns, determined from 15N relaxation data), which is in good agreement with the expected value for a folded 7-kDa protein. The AIRE1-PHD1 solution structure was determined from a large number of restraints (an average of 17.4 per residue) using the program CNS (37) interfaced with ARIA1.2 (32). Experimental restraints and structural statistics over the 20 lowest energy structures are summarized in Table I. Residues 295–345 adopt a well-defined tertiary structure with an RMSD of 0.6 Å for backbone atoms and have all residues in the allowed regions of the Ramachandran plot. In contrast, the N- and C-terminal residues (amino acids 293–294 and 346–354) are disordered as indicated by the small number of NOEs and negative heteronuclear NOE values (see Supplementary Materials).

AIRE1-PHD1 folds into a compact globular domain made of extended loops, with a short two-stranded anti-parallel β-sheet (residues 308–311 and 316–319) and a C-terminal α-helix (residues 338–342) as the sole elements of secondary structure (Fig. 1C). Similar to other PHD finger structures (38–40), AIRE1-PHD1 has a zinc-dependent fold. Introduction of an excess of the metal-chelating agent EDTA (20 mM) led to complete unfolding of the protein as indicated by the loss of chemical shift dispersion in the 1H-1D (Fig. 5D) and HSQC spectra (see Supplementary Materials). Removal of EDTA and the addition of two equivalents of zinc results in the refolding of the domain, thus demonstrating that the uptake of zinc is totally reversible and crucial for proper folding. The presence of Zn2+ and the stoichiometry (protein:metal 1:2) were also confirmed by atomic absorption. The two Zn2+ ions, located at opposite ends of the β-sheet (Fig. 1C), are separated by 15 ± 0.5 Å and are coordinated by conserved residues in a cross-brace manner, similar to that of the RING domain and in contrast with the sequential coordination found in LIM domains (39) (Fig. 2). The first zinc binding site comprises residues Cys329, Cys332, His319, and Cys322; similar to other PHD fingers the histidine coordi-

nations occur through the Nδ1 position of the imidazole ring. The Nε position is protonated as judged from chemical shifts in a two-dimensional 1H-15N long-range HMOC spectrum. The Hε proton of the histidine ring has a characteristic downfield shift (11.2 ppm) and is possibly involved in a hydrogen bond with the backbone carbonyl of Gly305. The latter observation explains why the histidine imidazole Hε exchanges sufficiently slowly to be observed in one-dimensional and two-dimensional spectra recorded in H2O. The second zinc coordinating site is composed by the thiol groups of Cys311, Cys314, and Cys337, Cys340 which are respectively located on the loop connecting the two β-strands and on the C-terminal α-helix (Fig. 1C).

The domain is further stabilized by a network of hydrophobic

Table I

| Summary of conformational constraints and statistics for the 20 best structures of AIRE1-PHD1 |
|---|---|
| Restraints used for structure calculations<sup>a</sup> | <SA> |
| All | 970 |
| Sequential (i−j = 1) | 213 |
| Medium range (1 < i−j ≤ 4) | 59 |
| Long range (i−j > 4) | 219 |
| Intraresidual | 488 |
| Hydrogen bonds | 6 |
| Dihedral angles | 66 |
| Residual dipolar couplings | 44 |
| Q-factor for experimental residual dipolar coupling restraints<sup>d</sup> | 0.11 ± 0.01 |

<sup>a</sup> No distance restraint in any of the structures included in the ensemble was violated by more than 0.4 Å.

<sup>b</sup> No dihedral angle restraints in any of the structures included in the ensemble was violated by more than 5°.

<sup>c</sup> No distance constraint in any of the structures included in the ensemble was violated by more than 0.4 Å.

<sup>d</sup> Quality factor for the RDC refinement (54).

<sup>e</sup> RMSD between the ensemble of structures <SA> and the mean structure.

<sup>f</sup> RMSD between the ensemble of structures <SA> and the mean structure.

<sup>g</sup> <SA> refers to the ensemble of the 20 structures with the lowest energy.

<sup>h</sup> Structural quality

| % Residues in most favored region of Ramachandran plot | 87.40% |
| % Residues in additionally allowed region | 12.60% |

<sup>i</sup> Deviation from idealized covalent geometry

| Bonds (Å) | 0.001 ± 0.0001 |
| Angles (°) | 0.299 ± 0.004 |
| Coordinate Precision (Å) | 0.65 ± 0.11 |
| All heavy atoms (residues 295–345) | 1.41 ± 0.16 |
| N, Cα, Cβ in secondary structure | 0.37 ± 0.064 |
| All heavy atoms in secondary structure | 0.98 ± 0.12 |

<sup>j</sup> No distance restraint in any of the structures included in the ensemble was violated by more than 0.4 Å.

<sup>k</sup> No dihedral angle restraints in any of the structures included in the ensemble was violated by more than 5°.

<sup>l</sup> RMSD between the ensemble of structures <SA> and the mean structure.

<sup>m</sup> Quality factor for the RDC refinement (54).

<sup>n</sup> No distance constraint in any of the structures included in the ensemble was violated by more than 0.4 Å.

<sup>o</sup> No dihedral angle restraints in any of the structures included in the ensemble was violated by more than 5°.
interactions between well conserved residues such as Leu<sup>208</sup>, Ile<sup>209</sup>, Phe<sup>215</sup>, Leu<sup>230</sup>, Leu<sup>233</sup>, Leu<sup>227</sup>, and Trp<sup>235</sup> (Fig. 1A). Classical PHD fingers are characterized by the presence of three variable loops L1, L2, and L3 comprising residues 303–307, 315–317 and 328–334, respectively, in AIRE1. Overall the three loops are well defined by the ensemble of structures; only residues 330–334 located on L3 appear less ordered, in agreement with the paucity of inter-residual NOEs observed for these residues (Fig. 1B). Consistently, residues around Gly<sup>323</sup> show increased values of both T<sub>1</sub> and T<sub>2</sub> relaxation times and a reduction of the heteronuclear NOE intensities (see Supplementary Materials), thus indicating that the structural heterogeneity of residues loop L3 is due to internal motion on a rapid time scale (i.e. in the pico- to nanosecond range).

**Structural Comparison with Known PHD Finger Structures**—The three-dimensional structure of AIRE1-PHD1 was compared with previously determined PHD finger structures. Table II summarizes the RMSDs calculated for aligned residues and the Z-scores derived from the DALI search for structural neighbors, where a Z-score< 2.0 typically indicates a structurally dissimilar pair. Among the solved structures the PHD finger of the Mi2β transcription factor (40) displays the highest Z-score and the lowest RMSD over equivalenced residues when compared with AIRE1-PHD1. In contrast, the PHD finger of the Williams-Beuren Syndrome transcription factor (WSTF) (38), despite considerable sequence identity, shows the highest backbone RMSD (3.3 Å) probably because of the three amino acid insertion in loop L1 (Fig. 1A). AIRE1-PHD1, Mi2β, and WSTF all present a βαβ fold. In both AIRE1-PHD1 and Mi2β the helix starts just after loop L3 and is part of the second zinc coordinating site. Interestingly, some conformers of the PHD finger of the co-repressor KAP-1 display an additional β-strand preceding the second zinc binding site (comprising residues 664–666) (39), which is not present in the other PHD finger structures. In all four PHD structures the domain is stabilized by two zinc ions ~15-Å apart and by a network of hydrophobic interactions centered around a highly conserved tryptophan, a distinguishing feature of many PHD fingers (Fig. 1A).

The analysis of the electrostatic surface potential calculated for the AIRE1-PHD1 structure reveals a large negatively charged surface area and only a small positive patch on the opposite side (Fig. 3, A and B). This suggests that AIRE1-PHD1 is more likely to be involved in protein-protein interactions than in nucleic acid binding. Conversely, a homology model of the second PHD finger of AIRE1 (AIRE1-PHD2), which is well folded in solution (Fig. 5G), displays a positive electrostatic surface (Fig. 3, C and D).

**Structural Comparison with Known RING Finger Structures**—Several key features, such as the interleaved zinc coordination scheme and similar overall topology suggest that RING and PHD fingers are structurally and functionally related to each other (16). Recently, it was proposed that the PHD and RING fingers share a common function as E3 ligases in the ubiquitination pathway (16, 17). Therefore, we compared the AIRE1-PHD1 structure with the RING fingers of the well-known E3 ligases c-Cbl, and CNOT4 (41, 42). Despite similar topology and similar inter-zinc distances the structural alignments over equivalent residues give very low Z-scores (Z< 1) (Table II and Fig. 4, C and D), in contrast with the very close alignment of the AIRE1-PHD1 and Mi2β structures (Z-score =

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**Table II**

Structural comparisons between AIRE1-PHD1 and known PHD and RING finger structures according to DALI output

| Structure | Z-score | Equival. res. | Seq. identity | RMSD with AIRE1-PHD1 |
|-----------|---------|---------------|---------------|-----------------------|
| Mi2β (1MM2) | 7.4 | 49             | 53            | 1.6                   |
| KAP-1 (1FP0) | 3.2 | 42             | 33            | 2.6                   |
| WSTF (1F62) | 4.4 | 48             | 54            | 3.1                   |
| c-Cbl (1FBV) | 0.5 | 34             | 18            | 3.24                  |
| CNOT4 (1UR5) | 0.7 | 34             | 21            | 3.3                   |
| K3 (1VYX) | 0.8 | 35             | 31            | 3.1                   |

* PDB codes of the structures used for structural alignment with AIRE1-PHD1.
* Z-score, i.e., strength of structural similarity in standard deviations above expected as defined in Ref. 35.
* Total number of equivalenced residues.
* Percentage of sequence identity over equivalenced positions.
* Positional root mean square deviation of superimposed Ca atoms.
7.4) (Fig. 4A). In particular, in RING fingers the α-helix is involved in the coordination of the first zinc ion, whereas in AIRE1-PHD1 the C-terminal helix contains the last cysteine dyad coordinating the second metal binding site (Figs. 2 and 4). Both in c-Cbl and CNOT4 this helix is part of a shallow hydrophobic groove suitable for van der Waals’ interactions with their E2 partners. The structural alignment of AIRE1-PHD1 on CNOT4 and c-Cbl does not show a similar hydrophobic surface. Conversely AIRE1-PHD1 displays at the C terminus of L3 a tryptophan surrounded by hydrophobic residues, which is not present in RING fingers but aligns closely with the corresponding Trp in Mi2β (Fig. 4A). AIRE1-PHD1 was also compared with the N-terminal domain of the Kaposi’s sarcoma-associated Herpes virus (KSHV) protein K3, whose sequence was previously characterized as PHD finger (Table II, Fig. 3B). A recent report (43), however, clearly demonstrates that K3 is a variant member of the RING family, with structural features similar to CNOT4. Indeed, AIRE1-PHD1 displays a very low Z-score (0.8) with K3 and the structural alignment is poor (Fig. 4B). Together these observations suggest that although RING and PHD fingers are members of the treble clef class of zinc binding domain (44), the structural relationship between these RING domains and AIRE1-PHD1 is extremely low, suggesting that they do not share the same function.

**Structural Analysis of Pathological AIRE1-PHD1 Point Mutations—** Several APECED causing point mutations (V301M, C311Y, P326L, and P326Q) have been identified in the AIRE1-PHD1 sequence (3). To elucidate the structural impact of these substitutions we expressed, purified, and analyzed the corresponding AIRE1-PHD1 mutants by NMR spectroscopy. The 1H–1D spectra of the four mutants were compared with spectra of the wild-type PHD fingers (Fig. 5). The C311Y mutation (Fig. 5A), located on the second zinc binding site (Fig. 5H), impairs metal binding thus completely destroying the fold of the domain, as shown by the increased line width of the 1H–1D spectrum and by the loss of chemical shift dispersion, similar to what is observed for wild-type AIRE1-PHD1 after zinc removal (Fig. 5B).

The P326Q (Fig. 5C) and P326L (Fig. 5D) spectra display a residual chemical shift dispersion indicative of partial structure formation, confirmed by the presence of characteristic β-sheet long-range contacts in a NOESY spectrum (data not shown). However the 1H–1D NMR spectra of both the proline mutants exhibit fewer NMR signals at low field and an increase of intensity for random coil signals (i.e. between 7.6 and 8.4 ppm), indicative of partial disruption of the tertiary fold. Moreover, line broadening and the presence of additional peaks (Fig. 5, C and D) suggest the presence of different conformers, possibly due to peptidy1-protein cis-trans isomerization of Pro45925 and/or Pro45926. Notably, in the wild-type protein Pro45925 adopts the cis conformation (Fig. 5H), as assessed by characteristic Hα–Hα+1 NOEs. Most probably the mutation of Pro45926 also affects the conformation of Pro45925, thus destabilizing the region around these residues. Both prolines are located in the solvent-exposed variable loop L3 (Fig. 5, H and I), which has been suggested to play a functional role for the PHD finger (40). Therefore it is possible that their mutation may directly alter interactions with potential ligands.

In contrast with the other mutants, the V301M substitution does not appear to have any impact on the structure or on the stability of the domain (Fig. 5E), since its spectrum is almost identical to that of the wild type (Fig. 5F), displaying the characteristic sharp and well-dispersed signals of a folded protein. This result suggests that Val3311, which is partially solvent-exposed (Fig. 5, H and I) is likely to play a functional role rather than a purely structural role in the domain. The importance of this site is also evident from a structurally corresponding, pathological mutation (Trp to Ser) in the PHD finger of the α-thalassemia mental retardation syndrome (ATRX) protein (45).

We verified the zinc binding properties of the V301M, P326L, and P326Q mutants. As for the wild-type protein, the removal of zinc ions by EDTA led to the loss of chemical shift dispersion and unfolding of the domain, whereas upon reintroduction of zinc the spectra regained their original aspect with the characteristic peak at 11.2 ppm corresponding to the Hε of the zinc-coordinating histidine His319 (Fig. 5).

**Functional Studies of AIRE1-PHD1—** It was recently reported that AIRE1 mediates E3 ubiquitin ligase activity and that a region containing the first PHD finger is sufficient for this activity (21). We therefore tested if our AIRE1-PHD1 NMR construct has a similar function. Ubiquitination occurs via an enzyme cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase E3 (46). We used an in vitro cell-free system containing recombinant-purified E1, E2 (UbcH5B), 32P-labeled ubiquitin...
and ATP, in which E3 ligase activity was assessed by the ability to promote synthesis of polyubiquitin conjugates. Since the target of the putative E3 ligase activity of the PHD finger is unknown, this was a substrate-free assay in which auto-ubiquitinated forms of AIRE1-PHD1 should appear as a result of E3 ligase activity. Under a range of experimental conditions tested, we did not observe the formation of any polyubiquitin conjugates, neither for wild-type AIRE1-PHD1 protein (66 residues) nor for the fold destroying mutant C311Y, nor for longer wild-type AIRE1-PHD1-containing constructs (AP1 and AP2, 99 and 86 residues, respectively) that precisely matched the protein used by Uchida et al. (21) (Fig. 6). As a positive control, the assay was performed in the same conditions substituting AIRE1-PHD1 with MDM2, a RING finger containing protein with well-documented E3 ligase activity (47).

According to the intermolecular interfaces observed in the structures of the c-Blk/UbcH7 complex (41), the CNOT4/UbcH5B complex (42), and the K3/UbcH13 complex (43), direct interactions occur between the RING domains and their cognate E2. We therefore used NMR spectroscopy to search for direct interactions between AIRE1-PHD1 and its cognate E2 (UbcH5B, according to Ref. 21). We used two-dimensional $^1$H-$^{15}$N correlation experiments to monitor for spectral changes induced by binding. In a first experiment, we titrated unlabeled UbcH5B into $^{15}$N-labeled AIRE1-PHD1. In a second experiment, unlabeled AP1 was titrated into $^{15}$N-labeled UbcH5B. In neither case did we observe any spectral changes, demonstrating a lack of interaction between the PHD finger and UbcH5B (see Supplementary Materials).

A single report has also proposed that a number of PHD fingers may act as nuclear phosphoinositide receptors (15), with AIRE1-PHD1 potentially binding phosphatidylinositol 3-phosphate (PtdIns(3)P). However, in NMR binding experiments, which are able to detect even weak interactions with dissociation constants in the millimolar range, we could not observe any interactions between AIRE1-PHD1 and PtdIns(3)P.

**DISCUSSION**

AIRE1 is a multidomain protein harboring two PHD fingers, which are implicated in transcriptional activation and nuclear dot formation (4, 48–50). The PHD fingers of AIRE1 also have...
related to differences in surface electrostatics: of the known PHD finger structures, only AIRE1-PHD1 presents a single large cluster of negative residues on one face of the protein.

The suggestion that AIRE1 is an E3 ligase originated from the observation of sequence similarity between PHD and RING fingers, the latter being well known E3 ligases (16). While AIRE1-PHD1 shares some sequence and structural features with RING fingers (Figs. 1, 2, and 4), the overall structural similarity is rather low. In particular, AIRE1-PHD1 does not share the characteristic hydrophobic cleft that is used by RING fingers in E2 binding (41, 42). Furthermore AIRE1-PHD1, like other PHD fingers, exhibits a conserved buried tryptophan, which is absent in the RING structures (Figs. 1 and 4). Sequence and structure comparison studies concur that PHD and RING fingers are structurally distinct (19, 20). Sequence profile studies have also shown that those viral PHD fingers, which work as E3 ligases are merely variants of the RING family and should not be considered as bona fide PHD fingers (19, 20). This conclusion was confirmed by the recently determined structure of the N-terminal domain of the KSHV K3 protein, which revealed a RING domain fold (43).

We performed ubiquitination assays on several AIRE1-PHD1 constructs, but could not detect any E3 activity. Similarly, no interaction between AIRE1-PHD1 and its proposed cognate E2 was observed in NMR binding experiments, which can detect even low affinity interactions. Collectively, these results suggest that AIRE1-PHD1 is unlikely to be an E3 ubiquitin ligase. In agreement, the KAP-1 PHD finger shares high similarity with AIRE1-PHD1 (Fig. 1 and Table II) and does not exhibit E3 ligase activity (39).

The important role of PHD fingers is highlighted by the occurrence of pathological PHD finger mutations that trigger diverse human disorders such as ATRX syndrome, Williams-Beuren syndrome (38), Rubinstein-Taybi syndrome, Borioss-Forsman-Lehmann syndrome, and myeloid leukemias (references in Ref. 39). Herein, the AIRE1-PHD1 structure allowed us to rationalize the impact of known PHD mutations associated with APECED disease. The AIRE1-PHD1 mutations generally fall into two categories: those affecting the structure and those that preserve the protein fold but probably affect its ability to interact with binding partners. Most notably, the C311Y mutation causes complete unfolding of the domain, possibly explaining why AIRE1 bearing this mutation fails to form nuclear dots (52), and confirming that the PHD structure is required for correct nuclear organization (7). Indeed, a very recent report has shown that the ACF1 PHD fingers (Fig. 1A) mediate histone binding (14), indicating a potential role in nuclear organization by facilitating nucleosome interactions. Future biochemical and structural studies aiming to verify the possible role of AIRE1 PHD fingers in histone binding should help to further elucidate the molecular functions of the AIRE1 protein.

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