AIRE’s CARD Revealed, a New Structure for Central Tolerance Provokes Transcriptional Plasticity*

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Developing T cells encounter peripheral self-antigens in the thymus in order to delete autoreactive clones. It is now known that the autoimmune regulator protein (AIRE), which is expressed in thymic medullary epithelial cells, plays a key role in regulating the thymic transcription of these peripheral tissue-specific antigens. Mutations in the AIRE gene are associated with a severe multiorgan autoimmune syndrome (APECED), and autoimmune reactivities are manifest in AIRE-deficient mice. Functional AIRE protein is expressed as distinct nuclear puncta, although no structural basis existed to explain their relevance to disease. In addressing the cell biologic basis for this supramolecular architecture, we were surprised to discover the mechanism of AIRE self-assembly into nuclear foci. At the time of this writing, no supramolecular structure has been described for any sub-nuclear domain; however, in vivo evidence for AIRE’s association with a global transcription cofactor, which may underlie AIRE’s focal, genome-wide, alteration of the transcriptome.

Central tolerance remains a conundrum for immunologists (1). At the cellular level, we know that depletion of self-reactive thymocytes is preceded by their thymic preview of tissue-specific antigens. This antigenic display and the transcriptional alterations required to generate it are attributed to the activity of a single protein, autoimmune regulator (AIRE).7 whose molecular mode of action remains largely unknown. The necessity for AIRE during the generation of tolerance is illustrated by the multiorgan autoimmune disease that results upon its mutation. This autoimmune syndrome, termed autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune-polyglandular syndrome type 1, is diagnosed when two of the following three symptoms are present: hypoparathyroidism, adrenal insufficiency, or chronic mucocutaneous candidal infections.

Microarray analyses have shown that the AIRE gene (2, 3) encodes an activity that imposes large scale alterations to the transcriptome of medullary thymic epithelial cells (mTECs) to favor transcripts of tissue-specific antigens (TSAs) (4, 5). Although mTEC expression of TSAs incurs protection from autoimmunity (6–8), the molecular mechanisms of AIRE function have been difficult to deduce. AIRE mutations in APECED patients are often of limited value in analyzing AIRE function as many generate truncates or no protein at all. However, a series of missense mutations in one mutation hot spot has been informative (9); the amino-terminal sequence labeled the homogeneously staining region (HSR), which drives AIRE oligomerization (9). However, “HSR” is a descriptive moniker and implies no structural information and this has hampered efforts to discover the mechanism of AIRE self-assembly into nuclear foci. At the time of this writing, no supra-molecular structure has been described for any sub-nuclear domain; however, in

The abbreviations used are: AIRE, autoimmune regulator; mTEC, medullary thymic epithelial cell; TSA, tissue-specific antigen; APECED, autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy; HSR, homogeneously staining region; CARD, caspase recruitment domain; CBP, cyclic AMP-response element-binding protein binding protein; RANKL, receptor activator of nuclear factor-κB ligand; SAND, Sp100, AIRE, NucP41/P75, DEAF-1; FTOC, fetal thymic organ culture; LT, lymphotoxin B; PML-ND, promyelocytic leukemia nuclear domain; DMEM, Dulbecco’s modified Eagle’s medium; DAPI, 4’,6-diamidino-2-phenylindole; PDB, Protein Data Bank.
AIRE’s CARD Drives Nuclear CBP Accumulation

many cases, the determination of the structural and cellular criteria for puncta assembly has been a critical milestone in determining function. This has proven to be the case for nuclear domains of promyelocytic leukemia protein (PML-NDs) that modulate transcriptional responses to stress (10).

Unlike AIRE, the protein assembly motifs that drive nucleation of PML puncta are evident if one examines the PML primary amino acid sequence. However, protein homologues that share identical folds can evade detection by standard sequence-based search algorithms if sequence identity is low (structural conservation may require few amino acid matches) (11). With this in mind, we took advantage of a powerful structure-based sequence alignment program to reveal previously hidden homologies in AIRE’s HSR (12). We now show that the amino terminus of AIRB expresses a previously undescribed caspase recruitment domain (CARD) whose integrity is needed for AIRE transactivation of TSAs. This finding presents fresh insights into how missense AIRE mutations found in APECED patients disrupt AIRE function to cause disease.

AIRE’s function in altering the transcriptome is likely to involve interactions with histone modifying activities. This possibility has been raised by in vitro experiments showing that AIRE interacts with a global transcription cofactor, the histone acetyltransferase, cyclic AMP-response element-binding protein (CBP) to enhance AIRE’s transactivating potential (13, 14). However, the biologic relevance of these findings is questionable given other studies showing CBP sequestration by PML-NDs (14, 15). In vitro experiments are with 0.1 μg of reporter constructs, 0.3 μg of AIRE constructs or the control (pCDNA 3.1 B myc/his), and 0.5 μg of CBP. For endogenous promoter activation assays, 0.5 μg of AIRE construct or control DNA (pCDNA 3.1−B myc/his) with 0.9 μg of CBP (pRc/RSV-mCBP-HA-RK, a gift from R. Goodwin, Oregon Health and Science Institute) were transfected. cDNA samples were analyzed in triplicate by quantitative PCR using the qPCR™ SYBR® Green Core kit (Eurogentec) and ABI Prism 7900HT. The relative gene expression levels were calculated using comparative Ct (ΔΔCt) method (Applied Biosystems). The following primers were used for quantitative RT-PCR: for HPRT as an internal reference, HPRTexon6 and GACITTGCTTTCCCTGTCAAGHPRTexon7 AGTCTGGCTTATATCCAACACTTCCG. Primer sequences to amplify the involucrin and S100A8 cDNAs were as follows: hINV forward, GCCCTACTGTTAGCTGGTACCA, and hINV reverse, GGAGGACAGCTTTGAGGACT; S100A8 forward, CTCAAGTATACGAAAGGGTGCGACAG, and S100A8 reverse, CACGCCCATTTTATCACCAGAATGAG. Transactivation Assays—Luciferase reporter assays, RNA purification, quantitative RT-PCR, and immunofluorescence were performed as in Ref. 14. For luciferase assays, transfections were with 0.1 μg of reporter constructs, 0.3 μg of AIRE constructs or the control (pCDNA 3.1 B myc/his), and 0.5 μg of CBP. For endogenous promoter activation assays, 0.5 μg of AIRE construct or control DNA (pCDNA 3.1−B myc/his) with 0.9 μg of CBP (pRc/RSV-mCBP-HA-RK, a gift from R. Goodman, Oregon Health and Science Institute) were transfected. cDNA samples were analyzed in triplicate by quantitative PCR using the qPCR™ SYBR® Green Core kit (Eurogentec) and ABI Prism 7900HT. The relative gene expression levels were calculated using comparative Ct (ΔΔCt) method (Applied Biosystems). The following primers were used for quantitative RT-PCR: for HPRT as an internal reference, HPRTexon6 and GACITTGCTTTCCCTGTCAAGHPRTexon7 AGTCTGGCTTATATCCAACACTTCCG. Primer sequences to amplify the involucrin and S100A8 cDNAs were as follows: hINV forward, GCCCTACTGTTAGCTGGTACCA, and hINV reverse, GGAGGACAGCTTTGAGGACT; S100A8 forward, CTCAAGTATACGAAAGGGTGCGACAG, and S100A8 reverse, CACGCCCATTTTATCACCAGAATGAG. Mice—AIRE null and wild-type C57BL/6 mice were bred at the University of Tartu (Tartu, Estonia) and the University of Birmingham (Birmingham, UK), respectively, and all experiments were performed in accordance with UK Home Office regulations. Aire−deficient mice (C57BL/6) were generated at The Walter and Eliza Hall Institute (Melbourne, Australia).
NOD and C57BL/6 mice were maintained in the Biological Services facility of the Department of Pathology at the University of Cambridge. Thymi were removed and snap-frozen. 10-μm sections were cut using a cryostat and adhered to polysine-coated slides (BDH). Sections were immediately fixed with acetone prior to storage at −80 °C.

Thymic Organ Culture and AIRE Induction—Thymic lobes were dissected from E15 BALB/c embryos. Each lobe was placed at the air-medium interface on top of a 0.8-μm isopore membrane filter (Millipore, Watford, UK), supported by an Artiwrap sponge (Medipost Ltd., Weymouth, Dorset, UK). The filter and sponge were submerged in DMEM-10 (DMEM supplemented with 10% fetal calf serum, 1 mM nonessential amino acids, 10 mM HEPES, 5 × 10−5 M 2-mercaptoethanol, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin) in 10% CO2 at 37 °C. 1.35 mM 2-deoxyguanosine was added to the media for 6 days to deplete T cells and dendritic cells. Lobes were briefly washed in fresh DMEM, and 1 lobe per well of a Terasaki plate was incubated in 20 μl of DMEM-10 with 10 μg/ml anti-Rank antibody alone (R & D Systems) or with 10 μg/ml anti LTβR (4H8 WH2, Alexis) for 2 or 4 days. For the 4-day stimulation, the medium containing fresh antibodies was replaced after 2 days. A control group was cultured in DMEM-10 only. The lobes were then snap-frozen and cut at 6 μm thickness for histological analysis.

Immunofluorescence—Immunostaining of transfectants was as described previously (24). Immunostaining reagents were as follows: anti-PML (clone N19, Santa Cruz Biotechnology), antimurine AIRE (rabbit polyclonal, a kind gift from Professor Peltoton, Biomedicum, Helsinki, Finland), and anti-human AIRE (mouse monoclonal AIRE6.1 as described previously (23)). Acetone-fixed thymus sections were rehydrated in phosphate-buffered saline, permeabilized, and stained as above with the following: anti-PML (clone 36-1-104); anti-keratin 5 (Covance), anti-Aire (clone B1/02–5H12-2), and anti-CBP (clone C-20, Santa Cruz Biotechnology). Secondary antibodies used for transfectants and thymus sections were Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Cells and sections were mounted and counterstained with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA).

Microscopy—Micrographs of transfectants were obtained using a Zeiss Axioshot fluorescence microscope (100× oil immersion objective) and a DM IRBE Leica confocal microscope (Leica, Vienna, Austria) with a 63× 1.32 oil immersion objective or with or without Nikon Eclipse TE2000-U microscope (60× water-immersion objective). Confocal microscopy of FTOC sections was performed with a LSM10 Zeiss confocal microscope.

RESULTS

AIRE Contains an Amino-terminal CARD Domain That Is Required for Nuclear Puncta Formation—The AIRE1 gene encodes a 545-amino acid residue protein with a molecular mass of 58 kDa. Previous sequence-based analyses had identified four distinct protein domains as follows: an amino-terminal homogeneously staining region (HSR) formed by the first 100 amino acids of AIRE, two plant homeodomain (PHD) fingers, as well as an Sp100, AIRE, NucP41/P75, DEAF-1 (SAND) domain (25). All but the first of these domains are implicated in interactions with chromatin and nucleic acids (9, 25). Key observations that led to our study of the HSR domain were that two-thirds of all of APECED-associated missense mutations are clustered in this domain (25), which is also the most highly conserved sequence in the AIRE protein (supplemental Fig. 1). These observations suggest that the HSR is vital for AIRE’s function. Primary sequence-based data base searches identify AIRE’s homology to the Sp100 protein family (supplemental Fig. 2) (26), which contains the HSR-SAND domain structure, suggesting a common evolutionary ancestor (27). As for AIRE, the HSR domain of Sp100 proteins can drive their oligomerization to form nuclear puncta (28, 29). Despite this homology, the HSR nomenclature is entirely nonfunctional (30), and this domain remains structurally uncharacterized.

Disorder prediction (31) of full-length AIRE suggested that the HSR is ordered with a high propensity to assemble as a globular folded domain, whereas the remaining protein showed regions of disorder and flexibility, a feature commonly found in proteins that interact with nucleic acids (Fig. 1A). To further analyze the amino-terminal HSR domain, a three-dimensional structure-based sequence analysis was utilized (12) allowing the identification of homologous proteins from their conserved three-dimensional structures, even in the absence of high primary sequence identity. In this case, a sequence-structure homology recognition analysis indicated that the HSR region may correspond to a previously uncharacterized CARD domain. The target AIRE sequence was found to align well with the CARD domain from Apaf-1 (apoptosis-activating factor 1) (Protein Data Bank code 1ygs_p). This alignment was subsequently extended to include the sequences of three additional CARD domains with fully characterized three-dimensional structures (Fig. 1B).

A key feature of the primary sequences of the CARD family is a series of highly conserved hydrophobic residues that form the core of the folded domain. The six amphipathic α-helices that constitute the secondary structure of the CARD domain are presumed to fold around this cluster of hydrophobic residues, all of which are conserved in solved CARD domain structures (Fig. 1B). This pattern of residues, because of both their importance in defining the CARD fold and their high level of conservation, can be identified as the signature of this protein family. Combining the AIRE/Apaf-1 alignment with the CARD family alignment indicated that this CARD signature is present in the amino-terminal region of AIRE giving a very strong indication as to the accuracy of the FUGUE assignment (Fig. 1B).

This detailed multiple sequence alignment was used to create a hypothetical model of the AIRE CARD domain structure using homology modeling techniques. Importantly, the CARD model was found to fully satisfy spatial and stereochemical restraints presenting a PROCHECK G-factor of −0.07 and with 94.5% of residues found in the favored regions (and 0% in the disallowed regions) of a Ramachandran plot.

The model generated exhibits all of the requisite features of a CARD domain with 6 α-helices presented with Greek key topology along with spatial conservation of the key hydrophobic core residues (Fig. 2A). Furthermore, the domain shows the polarized distribution of electrostatic surface charges charac-
AIRE’s CARD Drives Nuclear CBP Accumulation

**FIGURE 1.** AIRE contains an amino-terminal CARD domain. A, PONDR VL-XT prediction for AIRE protein showing a structured amino terminus. PONDR scores below the boundary (0.5) signify a propensity for order, with scores above 0.5 indicating a high likelihood of disorder. B, structure-based sequence alignment of AIRE (residues 8–96) with the sequences of four other CARD domains that have known three-dimensional structures. APAF1, Apaf-1 (PDB code 3YGS_P); CASP9, caspase-9 (PDB code 3YGS_C); ICBR, iceberg (PDB code 1DGN); and RAIDD, RIP-associated ICH1/CED3 homologous protein with death domain (PDB code 3CRD). Amino acid residue numbers are indicated for each sequence. The key hydrophobic residues that constitute the core of these domains when correctly folded are shown in red. Yellow boxes indicate the positions of the six α-helices of the Apaf-1 CARD characteristic of the topology for this domain. The position of the putative CARD domain in the AIRE polypeptide is indicated in the schematic shown below the alignment, which also indicates positions of the SAND domain, two PHD fingers, LXXLL motifs, and nuclear localization signal (NLS).

Superimposition of the AIRE CARD model with the structures of the Apaf-1 and ICEBERG CARDs reveals the striking structural conservation (Fig. 2C). The amino-terminal domain of AIRE has been described previously as vital for the transactivation function of the protein and specifically for its oligomerization (29). As confirmation, an AIRE construct devoid of the CARD domain but still expressing a nuclear localization sequence, pGFP-AIREΔCARD, was transiently transfected into HeLa cells. Ablation of the CARD domain led to a diffuse staining phenotype with both cytoplasmic and nuclear expression evident (Fig. 2D).

Our assignment of a CARD domain to the amino terminus of AIRE was also supported by AIRE expression data gathered from transient transfectants (supplemental Fig. 3A). HeLa cells transfected with GFP-AIRE showed nuclear punctate, cytoplasmic filament, and aggregate fluorescence patterns, all consistent with previous reports (31), and distributions that contrast with in vivo staining patterns that are exclusively nuclear punctate (supplemental Fig. 3B). Overexpression of death-fold superfamily-containing proteins, which includes the CARD domain, commonly results in the nucleation of artificial death effector filaments (34) at intermediate filaments and microtubules (15). The cytoplasmic filaments of AIRE protein that we detect in cell culture are therefore likely to be death effector filaments, an assertion strengthened by their known association with microtubules and intermediate filaments (15).

**Correlation of CARD Domain Disrupting Mutants with Transactivation**—The availability of a structural model of the AIRE CARD domain permits us to analyze disease-associated APECED missense mutations. The positions of the 12 AIRE CARD residues that are mutated in APECED were mapped onto the CARD model and were found to lie either on the surface of the domain, where their substitution may disrupt protein/protein interactions, or at the hydrophobic core, which may lead to incorrect folding and inactivation (Fig. 3A).

Three APECED-associated substitutions, leucine 28 to proline (L28P), leucine 29 to proline (L29P), and lysine 83 to glutamate (K83E), were further analyzed for their effects on AIRE distribution and transactivation potential. These mutations were chosen as they lie either at the core (Leu-28 and Leu-29) or surface (Lys-83) of the CARD domain (Fig. 3B). Expression of the L28P or L29P mutants leads to a diffuse nuclear pattern of AIRE, contrasting with the punctate distribution characteristic of the wild-type AIRE protein (Fig. 4A). The K83E mutant exhibits an intermediate distribution with a speckled nuclear pattern. In keeping with our model showing that homotypic CARD domain interactions drive AIRE puncta formation, mutations at Leu-28 and Leu-29 have been shown to prevent AIRE self-association (9), whereas K83E has an intermediate phenotype that is more severe when analyzing ectopic versus endogenous promoters.
The effects of these mutations on AIRE’s ability to initiate the expression of endogenous and ectopic gene promoters were then studied. As ectopic gene targets we used the luciferase gene under the control of the full-length interferon-β and involucrin promoters. From analyses of AIRE-deficient mice, it was known that the endogenous genes involucrin and S100A8 are down-regulated in an AIRE null background (GEO Data Sets, record GDS2274), and hence these genes were used to assay endogenous gene transcription. We also transfected each AIRE construct with or without co-transfection of CBP followed by measurements of luciferase activity or real-time quantitative PCR (qRT-PCR) analysis of transcript levels. In each case, we found that the Leu-28 and Leu-29 mutations prevented transactivation, regardless of CBP expression (Fig. 4B), whereas the effects of the K83E mutation were minimal. Notably, wild-type AIRE-induced transactivation was always augmented by CBP.

From our CARD model the two Leu-Pro mutations would be predicted to partially or entirely unfold the CARD domain,
abrogating its function as a protein/protein interaction module. In contrast, the K83E mutation is predicted only to alter the charge distribution on the domain surface and not to disrupt the overall fold of the domain. These predictions were tested using software capable of estimating the level of structural destabilization encoded by point mutations. Site-directed mutator (21, 35) uses a statistical potential energy function that predicts the effect that a specific amino acid substitution may have on the stability of that protein and produces a score analogous to the free energy difference between a wild-type and mutant protein. The L28P and L29P substitutions were shown to induce structural alterations with corresponding pseudo-
\begin{equation}
\Delta G = 3.75 \text{ kcal/mol, implying overall gross destabilization of the CARD domain (a value of less than } -2 \text{ from this analysis indicates a large and general fold destabilization). On the other hand, the K83E mutation was predicted to be partially stabilizing, with a pseudo-
\end{equation}
\begin{equation}
\Delta G = 0.29 \text{ kcal/mol. These results emphasize the excellent overall correlation between the modeling and experimental analyses of these APECED-associated mutants.}
\end{equation}

**RANK Stimulation Promotes the Nuclear Co-localization of CBP and AIRE**—Given that AIRE’s CARD drives puncta formation, which indicates a transactivation-competent protein, we next addressed the question of whether AIRE protein co-localizes with the global transcriptional activator, CBP, *in vivo*. Synchronous AIRE induction required our use of an agent naturally involved in AIRE induction. Anti-lymphotoxin β-receptor (LTβR) antibody, a previously described inducer of AIRE-positive mTEC development (36), and anti-RANK (receptor activator of nuclear factor-κB antibody), a newly described AIRE agonist (16), were used to induce endogenous AIRE expression in cell lines. However, no AIRE induction was achieved following incubation of mTEC-derived cell lines with either the anti-LTβR or anti-RANK antibodies. Additionally, in these lines endogenous AIRE was dispersed and not punctate (data not shown), which would suggest that cell architecture is important for AIRE induction. In consequence we used *ex vivo* fetal thymic organ culture (FTOC) in which to test AIRE induction as this experimental system more closely resembles the intimate cell/cell contacts achieved in thymic architecture.

Dual immunostains for AIRE and keratin 5 (a marker specific for the thymic medulla) revealed that AIRE induction was only achieved by anti-RANK stimulation (Fig. 5A and supplemental Fig. 4). Prior to induction of AIRE, the distribution of CBP was predominantly cytoplasmic and punctate, a distribution that has been described in oocyte development (37). After induction, focal CBP and AIRE were observed in nuclei, with cytoplasmic CBP puncta clustered around AIRE negative nuclei (Fig. 5B). Nuclear puncta of CBP were excluded from the DAPI bright portions of nuclei that typify heterochromatin territories. These data suggest that RANK stimulation drives the focal nuclear accumulation of both CBP and AIRE. Interestingly, immunostains for PML protein in FTOC revealed unusually low numbers of PML-NDs both before and after RANK treatment, with no overt sequestration of CBP (Fig. 5, A and B).
Nuclear Accumulation of CBP following RANK Stimulation Requires AIRE Expression—To examine whether AIRE expression was requisite to achieve a nuclear concentration of CBP, FTOCs derived from AIRE knock-out versus wild-type mice were generated. The fetal thymi were treated with anti-RANK antibody, anti-RANK and anti-LTβR antibodies, or anti-LTβR antibody alone. In thymi taken from wild-type mice, AIRE induction was achieved solely with anti-RANK treatment. Furthermore, following this treatment, there was prominent nuclear staining of CBP and AIRE. Conversely, in thymi derived from AIRE knock-out animals, no nuclear accumulation of CBP (or AIRE) was detected following anti-RANK stimulation (Fig. 6) indicating the necessity for AIRE expression in the translocation of CBP into the nucleus.

DISCUSSION

These data address two critical aspects of AIRE function as follows: oligomerization into transactivation-competent complexes and association with the histone modifier CBP. Replacement of the non-functional designation, HSR, with CARD immediately suggests that AIRE is part of a novel tolerance-inducing molecular complex, almost certainly involving other CARD-expressing proteins. There is a growing acceptance of the importance of CARD domains in the formation of signaling machines that trigger apoptosis, inflammation, and innate immune recognition (32, 38–40). CARD domain proteins neither act solely as caspase recruitment and activation machines nor do they reside exclusively in the cytoplasm to regulate the activity of CARD-containing complexes (40–42). Future work will establish the protein partners of AIRE’s CARD domain.

The presence of acidic and basic charge clusters on opposing exposed surfaces of AIRE’s CARD moiety is predicted to drive self-association and interaction with other CARD domains, as exemplified by the caspase9-Apaf-1 CARD complex structure (32, 43). Homotypic interaction of CARD domains is typically used to orientate protein scaffolds (32) and to activate effector proteins, although homo-oligomers of CARD domains are less common than death or death-effector domain dimers (32). Our structural data were collected using human AIRE sequences, and alignment of the murine and human proteins show that sequence conservation is greatest at the amino terminus. Critically, all the residues identified in our alignment as being critical to maintain the CARD fold are conserved in the mouse sequence, as well as in other metazoans that express AIRE. These conserved residues are also the targets for mutation in APECED, which underlines their importance for AIRE function. When three such APECED-associated mutants were analyzed biochemically, an excellent correlation was noted between the predicted structural severity of the sub-
stition and their effects on subcellular localization and transactivation. The L28P and L29P substitutions were expected to be highly destabilizing and to potentially unfold the CARD domain, thereby abrogating the function of AIRE protein, and indeed, the transactivation activity of these mutants was found to be null. The K83E mutant, however, was predicted to be stable and to only affect the charge distribution on the CARD domain surface. When analyzed experimentally, these predictions were borne out by this mutant having residual transactivation activity and retaining the ability to form nuclear puncta.

Given the functional role of CARD domains, it is probable that this mutation causes APECED by disrupting electrostatic protein/protein interactions between AIRE and unidentified interaction partners. It is unfortunate that genotype/phenotype associations have not been reported for APECED patients other than the mild phenotype (hypothyroidism alone) found in patients expressing the founder mutation in Iranian Jews with APECED, Y85C. Y85C is, however, somewhat analogous to K83E in that it lies at the surface of the CARD domain, where it would not alter the CARD fold but potentially disrupt specific protein/protein interactions.

Our modeling data allow us to reassign CARD domains to both AIRE and its structural relatives, the Sp100 family proteins (26). Sp100 proteins are the alternatively spliced forms of a single gene and appear to regulate chromatin structure and transcriptional activity. The prototypic member of this family was the first PML-ND cargo protein to be identified (Sp100), and its ability to oligomerize has been attributed to the amino-terminal sequence that we now know contains a CARD domain. The identification of another CARD domain expressing protein within the PML-ND, caspase-2 (41), could implicate PML-NDs as scaffolds for caspase-2-mediated cell death, perhaps using Sp100 as an intermediary. If this is the case, then this effect must be restricted to particular cell types as we have surveyed caspase-2 expression in several transformed and untransformed cell types and find it to be largely constrained to the Golgi apparatus (data not shown). Given that PML-NDs generally repress the function of their cargo proteins, it would appear more likely that PML-ND resident caspase-2 is held in an inactive form. The identification of a CARD domain in Sp100 proteins may be of more relevance in regulating their antiviral functions. Sp100-B, -C, and -HMG repress the herpes simplex virus type-1 protein ICP0, but only when it is dispersed from PML-NDs (44). On the other hand, Sp100-A is permissive for ICP0 expression, which may encourage viral replication at the site of PML-NDs. All these Sp100 proteins express amino-terminal CARD domains, and their scaffold partners within and around PML-NDs may direct their immune regulatory activities.

The molecular details of AIRE induction via RANK signaling are not yet known, although the spectrum of defects seen in RANKL and RANK null mice (osteoporosis, defective T and B cell maturation, and failed lactation) suggest the lack of a differentiation stimulus (45, 46). In this case, RANK signaling may drive mTEC differentiation, which is in agreement with the description of multiple mTECs lineages (47). A differentiated cell status is associated with reduced PML-ND numbers, which may also be cytoprotective given the role of these structures in detecting injury and engaging apoptosis (48).

AIRE’s CARD Drives Nuclear CBP Accumulation

Neither the cytoplasmic distribution nor the large size of CBP puncta in mTECs were expected as CBP has been shown to be nuclear, forming puncta that approach the resolution limits of the microscope (~200 nm diameter) (49). Previous experiments had questioned epitope availability for CBP sequestered to puncta versus the nucleoplasmic fraction (50). As we were interested in any flux between nucleoplasmic CBP and that fraction localized to AIRE puncta, we first confirmed that our CBP antibody could stain both these fractions in AIRE transfectants (data not shown). These experiments showed that AIRE puncta and CBP could co-localize, which we subsequently confirmed in our organ culture experiments. The identification of cytoplasmic CBP puncta was uniquely made in our organ culture experiments. The identification of cytoplasmic CBP puncta was uniquely made in organ culture experiments and suggests that cytoplasmic CBP oligomerizes or is sequestered to unknown cytoplasmic structures. CBP entry to the nucleus is likely to be tightly regulated and may be coupled to the developmental plan of embryogenesis. The rarity of PML-NDs in mTECs also illustrates how monolayer cell culture experiments, in which numerous PML-NDs out-compete AIRE puncta for CBP, are inappropriate models with which to study mTEC function.

The nuclear concentration of CBP that occurs following RANK stimulation provides both an elegant mechanism for AIRE function and obviates any need to recruit CBP from other nuclear compartments as has been proposed (14). The finding that AIRE expression is requisite for the translocation of CBP from the cytosol to nucleus is intriguing. One could speculate that AIRE-deficient mTECs lack the cellular architecture and signaling hardware needed to trigger the nuclear accumulation of CBP, resulting in a key transcriptional activator failing to reach its nuclear substrates in AIRE null mTECs. From the transactivation data, this study has shown convincingly that co-expression of wild-type AIRE and CBP accelerates AIRE’s transactivation potential. Consequently, the nuclear accumula-
cion of CBP and AIRE in anti-RANK-stimulated nuclei may drive focal induction of tissue-specific transcripts.

The regulation of AIRE expression by RANKL is consistent with reports that this member of the tumor necrosis factor superfamily can activate NF-κB2 (also named p100) (51) and that NF-κB2 is essential for the expression of AIRE in mTECs (52). In conclusion, our model for thymic expression of TSAs as regulated by AIRE protein is postulated to require RANKL activation of NF-κB2 leading to AIRE and CBP co-accumulation in the nucleus, an activity driven by CARD-dependent interactions and resulting in large scale alterations in the mTEC transcriptome (Fig. 7).

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