Subcellular Expression of Autoimmune Regulator (AIRE) is Organized in a Spatiotemporal Manner

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Running Title: Molecular Dynamics of AIRE
SUMMARY

Autoimmune regulator (AIRE) is responsible for the development of organ-specific autoimmune disease in a monogenic fashion. Although AIRE has been demonstrated to play important roles in the establishment of self-tolerance, the molecular mechanisms controlling this action remain largely unknown. Rare, and low levels of, tissue expression together with the lack of AIRE-expressing cell lines have hampered a detailed analysis of the molecular dynamics of AIRE. Here, we have established cell lines stably transfected with AIRE and studied the regulatory mechanisms for its subcellular expression. AIRE existed in the nucleus in the established cell lines, appearing in a speckled form known as a nuclear body (NB), and we found that NB formation by AIRE was dependent on the cell cycle. Biochemical fractionation revealed that a significant proportion of AIRE is associated with the nuclear matrix, which directs the functional domains of chromatin to provide sites for gene regulation. Upon proteasome inhibition, AIRE NBs were increased with concomitant reduced expression in the cytoplasm, suggesting that subcellular targeting of AIRE is regulated by a ubiquitin-proteasome pathway. We also found that AIRE NBs compete for CREB-binding protein (CBP)/p300, a common coactivator of transcription, with promyelocytic leukemia gene product (PML), which may constitute a
novel regulatory mechanism of AIRE activities determined by functional association with other transcriptional regulating factors. These results suggest that the transcriptional regulating activities of AIRE within a cell are controlled and organized in a spatiotemporal manner.
INTRODUCTION

Breakdown of self-tolerance is considered to be the key event for the development of autoimmune diseases (1). Although a propensity to appear in families is one of the common features of autoimmune diseases, only a few genes relevant to the pathogenetic processes that underlie the development of autoimmune diseases per se are actually known (2). One of these genes is the autoimmune regulator (AIRE), whose mutation is responsible for the development of autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy (APECED: OMIM 240300) with autosomal recessive inheritance (3-6).

The AIRE gene encodes a predicted 58-kDa protein carrying a conserved nuclear localization signal, two plant homeodomain (PHD)-type zinc fingers, four LXXLL motifs or nuclear receptor interaction domains, and the recently described homogenously staining region and SAND domains (3,4). Based on the fact that PHD resembles the RING finger, which functions as an E3 ubiquitin (Ub) ligase (7), in both sequence and structure (8,9), we have recently found that AIRE acts as an E3 Ub ligase through the N-terminal PHD domain (PHD1) (10). The significance of this finding was underscored by the fact that disease-causing missense mutations in the PHD1 (i.e., C311Y and P326Q) abolished its E3 ligase activity. We speculate that AIRE might exert its
function by facilitating polyubiquitylation of substrate(s) in yet
undetermined processes.

One of the key features of AIRE in the context of autoimmunity is
its limited tissue expression in medullary epithelial cells and cells of the
monocyte-dendritic cell lineage of the thymus (11,12), both cell types
are considered to play major roles in the establishment of self-tolerance
by eliminating autoreactive T cells (1). Another characteristic feature
of AIRE is its subcellular localization. Immunocytochemical staining has
revealed its presence in the nucleus, appearing as a speckled pattern
known as a nuclear body (NB) (11-13). Despite intense efforts, a
coherent explanation of NB function has yet to emerge.

Although understanding the relationship between AIRE gene
malfunction and the breakdown of self-tolerance promises to help
unravel the pathogenesis of not only APECED, but also other types of
autoimmune disease, rare and low levels of tissue expression have
hampered a detailed analysis of the molecular dynamics of AIRE. This
situation is compounded by the lack of availability of established cell
lines constitutively expressing AIRE. In order to overcome these
difficulties, we have established cell lines stably transfected with AIRE
and studied the molecular dynamics of AIRE in detail, especially focusing
on the factors that influence its subcellular expression; establishment of
stably transfected cell lines with AIRE has enabled us to investigate many
aspects of AIRE which could not be easily addressed by the transient expression systems. Our results suggest the existence of a group of mechanisms that together control AIRE expression within a cell in a spatiotemporal manner.
EXPERIMENTAL PROCEDURES

Construction of the Fusion Gene for AIRE cDNA with Marker Genes

Human AIRE cDNA was amplified by PCR from Marathon-Ready human thymus cDNA (BD Biosciences, San Jose, CA). Briefly, AIRE cDNA was first amplified with adaptor primers according to the manufacturer's instructions, and then nested PCR was performed with AIRE-specific primers in which EcoRI and SalI restriction sites were added at the 5' and 3' ends, respectively. The following primers were used. AIRE-U1: CGGAATTCATGGCGACGGACGCGGCGCTAC; AIRE-D1: ACGCGTCGACTCAGGAGGGGAAGGGGGCCG. The resulting fragment containing the human AIRE open reading frame was ligated into the cloning site downstream of the enhanced fluorescent gene in pEGFP-C1 (BD Biosciences), FLAG-tag in pCR3 vector (Invitrogen, Carlsbad, CA) or 6Myc-tag in pCR3 vector. Site-directed mutagenesis was performed using human AIRE cDNA cloned into pBluescript II SK+ (Stratagene, San Diego, CA) as a template. pFLAG-AIRE C299A and C434A were generated with a Quick Change (Strategene) (10). The sequence of each constructed vector was confirmed by the dideoxy chain termination method with automated sequencing (Applied Biosystems, Foster City, CA).
Establishment of Stable Transfectants Expressing the AIRE Fusion Genes and Transient Gene Expression Studies

HeLa cells were transfected with pEGFP-AIRE plasmid by use of the FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Forty-eight hours later, culturing of the cells in selection media containing 400 g/ml of G418 (GIBCO BRL, Grand Island, NY) was started. After 14 days of culture in selection media, G418-resistant clones were isolated. The fluorescence signals from the cells were determined with the aid of fluorescence microscopy, and the representative fluorescent clones were further expanded. For the construction of retrovirus vector, human AIRE cDNAs were subcloned into pMX-puro-FLAG vector (kindly provided by Dr. T. Kitamura). Medullary thymic epithelial cell (mTEC) line 1C6 (kindly provided by Dr. M. Kasai) (14) or NIH3T3 cells were infected with the pMX-puro-FLAG-AIRE vectors, and cultured for 14 days in the presence of 2 g/ml of puromycin (BD Biosciences). For blockade of proteasome activity, cells were treated with 100 M N-acetyl-Leu-Leu-norleucinal (ALLN; Nacalai Tesque, Kyoto, Japan) or with 5 M Z-Leu-Leu-Leu-H (MG132; Calbiochem, La Jolla, CA) for 16 h. Transient gene expression studies were performed using the Lipofectamine transfection reagent (GIBCO BRL) for COS-1 and RD cells, and
electroporation for NB4 cells. The pSI-AIRE and CMX-PML expression plasmids and anti-AIRE Abs have been described previously (11,13).

**Isolation of Cellular Proteins and Western Blot Analysis**

Sequential isolation of subcellular components was performed as described previously (15), with a slight modification. Briefly, wild-type HeLa cells and pEGFP-AIRE-transfected HeLa cells were washed once with PBS, and resuspended in 400 μl of ice-cold lysis buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2% NP-40 (Sigma-Aldrich, St. Louis, MO) and a mixture of protease inhibitors. The cells were vortex-mixed vigorously, and kept on ice for 10 min. The mixture was then centrifuged for 1 min at 5,000 x g, and the supernatant was harvested for the cytoplasmic protein. After isolation of the cytoplasmic protein, nuclear pellets were resuspended in 50 μl of cytoskeletal (CSK) buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 1 mM DTT and protease inhibitors) containing 100 U RNase-free DNase I (Roche Molecular Biochemicals), and incubated for 50 min at 30°C. Ammonium sulfate was then added to a final concentration of 0.25 M, and the samples were kept on ice for 5 min. After centrifugation, the supernatant was harvested (chromatin fraction), and the pellets were then extracted with 2 M NaCl in CSK buffer. After further centrifugation,
the supernatant was harvested (2 M NaCl wash), and the pellets were lysed with 8 M urea to provide the nuclear matrix fraction. Western blot analysis was performed as described previously (16) with anti-GFP polyclonal Ab (Invitrogen), anti-IκB polyclonal Ab and anti-lamin A/C mAb (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-AIRE polyclonal Ab was raised by immunizing rabbits with peptides corresponding to the C-terminus of human AIRE. In situ extraction of the subcellular components from stable transfectants grown on coverslips was performed with the same method as described above.

**Immunocytochemical Staining**

pEGFP-AIRE-transfected HeLa cells seeded on coverslips were subjected to immunocytochemistry. Briefly, cells on the coverslips were fixed with 4% paraformaldehyde, and then permeabilized with PBS containing 0.5% Triton X-100. After washing the cells with PBS, non-specific binding was blocked with 1% BSA in PBS. Cells were then incubated with appropriate Abs (anti-PML and anti-CBP/p300 polyclonal Abs were from Santa Cruz Biotechnology). Alexa 594-conjugated anti-mouse and anti-rabbit Abs (Invitrogen) were used for detection of the primary Ab. After staining with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Roche Molecular Biochemicals) in PBS, coverslips were mounted with ProLong Antifade (Invitrogen), and fluorescence signals were observed with the
aid of a fluorescence microscope or confocal microscope equipped with an argon laser.

**Assessment of Ubiquitylation of AIRE**

Proteins were expressed by transfecting expression constructs with the Myc-tagged human AIRE cDNA and HA-tagged ubiquitin cDNA into COS-7 cells (17). Immuno-precipitation with anti-Myc mAb (clone 9E10) and Western blot analysis with anti-HA polyclonal Ab, both from Santa Cruz Biotechnology, were performed as described previously (16,17). For proteomics analysis, human AIRE was expressed in 293T cells with a modified tandem affinity purification (TAP) system (18). Purified proteins were subjected to SDS–PAGE on a 9% gel, and the gel was stained with Coomassie brilliant blue. The bands were excised from the gel, and then in-gel digested with trypsin (Promega, Madison, WI). The molecular masses of the tryptic peptides were determined using a MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), and protein identification was performed with the MASCOT search engine (Matrix Science, London, UK).

**In Vitro Ubiquitylation Assay**

The production of full-sized recombinant AIRE and *in vitro* ubiquitylation assay were performed as described (10,17). In brief,
reaction mixtures (20 μl) containing 1 g of recombinant AIRE, 0.1 g of recombinant rabbit E1 (Boston Biomedica, Cambridge, MA), 1 μl of crude E. coli lysate containing human Ubc4, 0.5 U of phosphocreatine kinase, 1 μg of Ub (Sigma-Aldrich), 25 mM Tris-HCl (pH 7.5), 120 mM NaCl, 2 mM ATP, 1 mM MgCl2, 0.3 mM DTT, and 1 mM creatine phosphate were incubated for 2 h at 30°C. The reaction was terminated by the addition of SDS sample buffer containing 4% 2-ME and heating at 95°C for 5 min. Samples were resolved by SDS-PAGE on a 6% gel and then subjected to Western blot analysis with a mouse mAb to Ub (clone 1B3; MBL, Nagoya, Japan) and horseradish peroxidase (HRP)-conjugated rabbit polyclonal Ab to mouse immunoglobulin (Promega). Signals were detected with ECL (Amersham Pharmacia Biotech, Aylesbury, UK).
RESULTS

*Establishment of Stable Transfectants Expressing AIRE*

It has been demonstrated that AIRE is predominantly expressed in the medullary thymic epithelial cells (mTEC) with the characteristic morphology of NB (11-13). However, the low levels of expression together with the lack of established cell lines constitutively expressing AIRE have hampered a detailed analysis of the molecular dynamics of AIRE. In order to approach this issue, we constructed a fusion gene in which the AIRE gene was fused with a green fluorescence protein (GFP) marker, and obtained a stable transfectant of this fusion gene in HeLa cells of epithelial cell lineage (GFP-AIRE/HeLa). We first examined the expression of GFP-AIRE in HeLa cells with the aid of a fluorescence microscope. As reported for endogenous AIRE as well as for transiently expressed AIRE gene in cultured cells, GFP-AIRE was expressed primarily in the nucleus (Fig. 1A, left). In addition to the moderate nucleoplasmic expression, GFP-AIRE was found concentrated in discrete nuclear speckles of NBs. Unexpectedly, not all of the cells showed typical NB morphology, and approximately two-thirds of the cells displayed faint and homogeneous nuclear fluorescence without apparent NBs. The low frequency of occurrence of cells with typical NBs was not due to the mixture of GFP-AIRE-expressing cells with untransfected cells, since
limiting dilution culture twice did not significantly change the population of cells with NBs (data not shown, and see below). Most of the GFP-AIRE NBs, when observed, were not colocalized with endogenous PML bodies, as revealed by double immunocytochemistry (Fig. 1B).

It has been demonstrated that NB formation by PML, a prototypic NB protein that is thought to play a role in the control of apoptosis (19,20), is cell-cycle related; PML formed NBs at a significantly higher level in the G1 phase (21). We therefore suspected that the infrequent expression of NBs in the GFP-AIRE/HeLa cells might be in part due to a similar mechanism. With the use of time-lapse analysis, we have observed that NBs largely disappeared before mitosis, but quickly re-formed after cell division (Supplementary Movie 1), clearly indicating that NB formation by AIRE is cell-cycle dependent.

**Association of AIRE with the Nuclear Matrix**

Although the structural characteristics of AIRE protein suggest that it is a transcriptional regulating factor (13,22-24), biochemical data supporting this idea are still limited. In order to obtain further insight into the function of AIRE, we investigated its subcellular localization. Nuclear matrix, the insoluble skeletal framework of the nucleus, is a dynamic structural subcomponent of the nucleus that directs functional domains of chromatin to provide sites for the specific control of nucleic
acids (25). In order to test whether AIRE might exert a transcriptional regulating function in the nuclear matrix fraction, GFP-AIRE/HeLa cells grown on coverslips were sequentially extracted in situ with a detergent (CSK buffer), DNase I, ammonium sulfate and high salt, leaving behind the nuclear matrix. The efficiency of chromatin digestion and removal was assessed by the disappearance of DNA staining with DAPI, and the presence of nuclear matrix after this treatment was confirmed by staining with a mAb against lamin A/C, a core nuclear-matrix-associated protein (26) (Fig. 2A). The speckled pattern of GFP-AIRE NBs was still detectable after these extractions, suggesting that a significant proportion of AIRE protein is associated with the nuclear matrix. This finding was also supported by Western blot analysis, in which nuclear matrix protein was recovered with 8 M urea after high salt extraction and assayed for the presence of GFP-AIRE protein. We found significant amounts of GFP-AIRE protein in this fraction from GFP-AIRE/HeLa cells, but not from wild-type HeLa cells (Fig. 2B). Successful fractionation of the cytoplasmic and nuclear matrix proteins was ascertained by Western blot analysis with Abs specific for IκB and lamin A/C, respectively. Although we did not observe apparent fluorescence signals from the cytoplasm of GFP-AIRE/HeLa cells, the cytoplasmic fraction contained a certain amount of GFP-AIRE protein. This discrepancy could be due to differences in the sensitivities of the detection systems used.
Association of AIRE protein with the nuclear matrix was also observed in other cell types stably transfected with AIRE (i.e., FLAG-AIRE/mTEC cells and Myc-AIRE/NIH3T3 cells; see below) (Supplementary Fig. S1).

**Ubiquitin-Dependent Subcellular Targeting of AIRE**

It has been demonstrated that PML changes its subcellular localization in a proteasome-dependent manner; many PML NBs accumulated in nucleoli upon treatment with proteasome inhibitors, such as MG132 (27). We used GFP-AIRE/HeLa cells to examine whether the subcellular localization of AIRE is controlled by a similar proteasome-dependent mechanism. Upon proteasome inhibition with ALLN or MG132 (data not shown), we observed enhanced fluorescence signals from the cells (Fig. 1A, right); not only was there an increase in the percentage of cells exhibiting NBs, but there was also an increase in the size of each dot (Supplementary Movie 2). Of note, NBs accumulated in proximity to the nucleoli, where DAPI staining is faint with this treatment. In contrast to GFP-AIRE/HeLa cells, HeLa cells stably transfected with GFP alone showed homogenous fluorescence signals from both cytoplasm and nucleus (data not shown). Upon proteasome inhibition, the distribution pattern of GFP in these cells remained unchanged, although the fluorescence signals were moderately enhanced.

Since AIRE and PML protein are not colocalized in untreated GFP-
AIRE/HeLa cells, as described above, but both change their localization within the nucleus upon inhibition of the proteasome pathway (Fig. 1A and (27)), we attempted to determine whether AIRE and PML were colocalized after proteasome inhibition. Most of the cells, if at all, showed no colocalization of AIRE and PML after proteasome inhibition, as observed for untreated cells (data not shown). Thus, AIRE and PML do not largely share their metabolic pathways, although subcellular targeting of these NBs might be controlled by a similar proteasome-dependent mechanism.

We have also investigated proteasome-dependent subcellular targeting of AIRE in the mouse mTEC line 1C6 (14) stably transfected with FLAG-tagged AIRE (FLAG-AIRE/mTEC). In contrast to GFP-AIRE/HeLa cells, FLAG-AIRE/mTEC cells displayed scattered fine dots of AIRE not only in the nucleus, but also in the cytoplasm (Fig. 3A, a-c). Upon proteasome inhibition, AIRE in the cytoplasm was decreased and AIRE predominantly accumulated in the nucleus (compare Fig. 3A, d-f with Fig. 3A, a-c) (Supplementary Fig. S2A). Enhancement of FLAG-AIRE expression after MG132 treatment, as assessed by Western blot analysis, was much weaker compared with that in GFP-AIRE/HeLa cells (our unpublished observation). We also established NIH3T3 cells stably transfected with FLAG-tagged AIRE (FLAG-AIRE/NIH3T3: Fig. 3B) as well as with Myc-tagged AIRE (Myc-AIRE/NIH3T3: Fig. 3C). Upon treatment
of FLAG-AIRE/NIH3T3 cells with MG132, AIRE in the cytoplasm was
decreased and AIRE predominantly accumulated in the nucleus
(compare Fig. 3B, j-l with Fig. 3B, g-i) (Supplementary Fig. S2B); both the
size of dots within the nucleus and the numbers of NB were moderately
increased. Although AIRE NBs were concentrated in some area within
the nucleus upon proteasome inhibition, it was not in the nucleoli; AIRE
and nucleolin were not co-localized in Myc-AIRE/NIH3T3 cells (Fig. 3C,
d). Thus, proteasome-dependent subcellular targeting of AIRE is not
confined to some particular cell types and/or tags for detection (i.e., GFP,
FLAG and Myc). In contrast, the increased amount of AIRE protein after
proteasome inhibition was somewhat dependent on cell type; GFP-
AIRE/HeLa cells showed a more dramatic increase compared with FLAG-
AIRE/mTEC cells or FLAG-AIRE/NIH3T3 cells.

**AIRE is Subject to Modification with Ubiquitin**

In order to confirm that AIRE is under proteasome-dependent regulation
for its subcellular targeting, we have tested whether AIRE can be
ubiquitylated *in vivo*. To investigate this, Myc-tagged AIRE and HA-
tagged Ub were coexpressed in COS-7 cells (which do not express
endogenous AIRE as assessed by RT-PCR; data not shown), and the
ubiquitylation of AIRE was assessed by immuno-precipitation with anti-
Myc mAb followed by detection with anti-HA Ab (Fig. 4A). Although
plasmid expressing Myc-tag alone did not show any association with Ub, ubiquitylated AIRE was easily detected when Myc-tagged AIRE was used for the transfection, clearly indicating that AIRE is subject to modification with Ub *in vivo*. Furthermore, AIRE expressed in 293T cells exhibited various molecular masses identified by peptide mass fingerprinting, consistent with the presence of polyubiquitylated forms of AIRE (Supplementary Fig. S3).

We have recently demonstrated that AIRE acts as an E3 Ub ligase through the N-terminal PHD domain (PHD1) (10). Because many E3 ligases exert ubiquitylation of their own protein (self-ubiquitylation) as well as of specific target protein(s), we tested whether the ubiquitylation of AIRE is due to self-ubiquitylation by AIRE itself. To this end, an *in vitro* ubiquitylation assay was performed by mixing recombinant full-length AIRE with Ub, recombinant E1 and Ubc4 (E2) in the presence of ATP. We then prepared immuno-precipitates for AIRE from this total reaction mixture, and performed Western blot analysis with anti-Ub Ab. Although the total reaction mixture contained polyubiquitylated proteins, no polyubiquitylated proteins were observed from the immuno-precipitates for AIRE (Fig. 4B); total reaction mixture and immuno-precipitates for AIRE contained similar amounts of AIRE as revealed by the Western blot analysis with anti-His Ab. This result suggests that AIRE does not ubiquitylate itself. Rather, we speculate
that the E3 ligase activity of AIRE is mostly directed toward ubiquitylation of specific target protein(s), and that there should be another E3 ligase responsible for the ubiquitylation of AIRE. This hypothesis is supported by the fact that NIH3T3 cells expressing a PHD1 mutant (C299A) that lacks E3 ligase activity (10) showed changes in the subcellular localization of AIRE upon proteasome inhibition similar to that seen in cells expressing wild-type AIRE (Fig. 3B, p-r). The effect of proteasome inhibition was also independent of the PHD2 domain, because a PHD2 mutant (C434A) also showed similar subcellular targeting to that of wild-type AIRE-transfected cells (Fig. 3B, v-x) (Supplementary Fig. S2B). Similar results were obtained from FLAG-AIRE/mTEC cells (Supplementary Fig. S2A).

**Regulation of AIRE Activities Determined by Colocalization with Other Transcriptional Regulators of NB Morphology**

Using co-precipitation experiments, we have previously demonstrated that AIRE is associated with CBP/p300, a common coactivator of transcription (22). On the other hand, CBP/p300 has been demonstrated to colocalize with PML (28). Because AIRE and PML are not colocalized, as demonstrated in this study, but both NBs are colocalized with CBP/p300, we suspected that AIRE activities might be regulated and/or modulated in part by competition with PML for
CBP/p300. In order to test this hypothesis, we used RD cells, in which both AIRE and endogenous PML are undetectable. Immunofluorescence of untreated RD cells showed no discrete nuclear dot formation by CBP/p300 (Fig. 5A, a). Instead, CBP/p300 was found in a diffuse and/or speckled pattern. When AIRE was introduced into RD cells, endogenous CBP/p300 was recruited to the AIRE NBs, confirming the \textit{in vivo} association between AIRE and CBP/p300 (Fig. 5B, a-c). In turn, when PML was expressed in RD cells, similar translocation of endogenous CBP/p300 to the PML bodies was observed (Fig. 5B, d-f), as demonstrated previously (28). When AIRE and PML were introduced simultaneously, AIRE and PML were mostly observed in distinct NB structures (Fig. 5B, g-i), as expected. Of note, in cells doubly transfected with AIRE and PML, CBP/p300 predominantly colocalized with PML bodies (Fig. 5B, j-l) rather than with AIRE NBs (Fig. 5B, m-o). These results suggest that the activities of AIRE might be indirectly regulated by PML through competition for CBP/p300.

Preferential colocalization of CBP/p300 with PML bodies rather than with AIRE NBs was also observed in NB4 cells. Endogenous PML-retinoic acid receptor (RAR) (due to the chromosomal translocation) displayed a speckled pattern instead of typical PML bodies in NB4 cells (29); colocalization of CBP/p300 with PML was still observed in this speckled pattern, as demonstrated in Fig. 5A, b. When both AIRE and
wild-type PML were transfected, AIRE and PML were mostly observed in distinct NB structures (Fig. 5C, a), as observed in RD cells. Again, endogenous CBP/p300 showed colocalization with PML (Fig. 5C, b), but not with AIRE (Fig. 5C, c). Thus, although AIRE associates with CBP/p300, CBP/p300 preferentially colocalizes with PML rather than with AIRE when both NB proteins are available, at least in the NB structures. We do not see this, however, as excluding CBP/p300 as an AIRE cofactor. Rather, we speculate that competition for CBP/p300 by AIRE and PML might be a novel and unique mechanism for the control of AIRE function.
DISCUSSION

We have studied the molecular dynamics of AIRE, a putative transcription-regulating gene responsible for the hereditary type of the human autoimmune disorder APECED. Although AIRE-deficient mice exhibit an autoimmune phenotype similar to that seen in the human disease (30,31), and have provided us with some important insights into how the immune system discriminates between self and non-self with the help of AIRE (32), the molecular mechanism for these actions by AIRE still remains unknown. Because the lack of cell lines constitutively expressing AIRE in sufficient amounts has been the main reason for these difficulties, we first established cell lines stably transfected with GFP-AIRE that enable us to monitor the molecular dynamics of AIRE within a single cell. As expected, we have observed GFP-AIRE expression primarily in the nucleus, with the characteristic morphology of NB. Unexpectedly, however, not all of the cells showed typical NB morphology, and many cells displayed faint and homogeneous nuclear fluorescence without apparent NBs. With the use of time-lapse analysis, we have found that NB formation is regulated by the cell cycle. These results suggest that not all of AIRE is NB bound. Instead, we speculate that AIRE forms NB structures under certain cellular conditions.

It has been shown that many transcription factors bind to the
nuclear matrix (25). Our studies have demonstrated that a significant amount of AIRE protein, including that in its NB form, exists in the nuclear matrix fraction. This is an important biochemical finding that supports a role for AIRE as a transcriptional regulating factor. However, it remains to be determined whether AIRE NBs themselves are the major site of transcriptional regulation by AIRE. Furthermore, it is not yet clear which form of AIRE, its nucleoplasmic form or NB form, is mainly responsible for the physiological function of AIRE. It is equally possible that AIRE in NBs is functionally distinct from that existing homogenously in the nucleoplasm. In this respect, it is interesting to note that PML bodies have not been reliably shown to be the sites of transcription, nor do they contain chromatin or RNA, although nascent RNA is present in the immediate neighborhood of PML bodies and they might be linked to regulation of transcription (20,33). Thus, the functional significance of NB formation by AIRE requires further study.

Given that AIRE is a transcriptional regulator, targeting of AIRE into the nucleus and/or to a particular site within the nucleus should be a relevant process that determines the effect of AIRE. In the present study, we have suggested that ubiquitylation is an important mechanism that determines the subcellular targeting of AIRE. We have observed altered localization of NBs within the nucleus from GFP-AIRE/HeLa cells after treatment with proteasome inhibitor. In both FLAG-AIRE/mTEC and
Myc-AIRE/NIH3T3 cells, AIRE NBs were increased with concomitant reduced expression in the cytoplasm upon proteasome inhibition (Fig. 3). These results suggest that ubiquitylation of AIRE is a critical modification process that dictates subcellular targeting. It is reasonable to speculate that ubiquitylation, facilitating nuclear targeting of AIRE, is a positive regulatory process for AIRE function.

We have previously suggested that AIRE shuttles between the nucleus and the cytoplasm (13), and that this process appears to be under the control of CRM-1, a protein responsible for nuclear export (34); treatment of the cells transiently AIRE-transfected with leptomycin B (LMB), a CRM-1 inhibitor, resulted in preferential localization of AIRE dots within the nucleus (34). Unexpectedly, however, treatment of GFP-AIRE/HeLa cells with LMB reduced the numbers of NBs to some degree (our unpublished observation). We suspect that the difference in experimental conditions (i.e., transient vs. stable AIRE expression) led to the formation of a different AIRE protein complex. In the stable expression system, AIRE may form a protein complex with factor(s) whose subcellular localization is under the control of CRM-1, and are responsible for the degradation of AIRE protein; accumulation of such factor(s) within the nucleus as a result of LMB treatment would result in the loss of AIRE NBs.

The effect of proteasome inhibition on the recruitment of AIRE
onto NB structures may support the idea that most of AIRE is not NB-bound; a fraction of AIRE is located on the discrete subnuclear structures, the AIRE NBs, the rest being in the nucleoplasm with homogenous distribution. In this respect, it is interesting to note that FANCD2, a gene responsible for Fanconi anemia, is mono-ubiquitylated in response to DNA damage and is targeted to nuclear foci (dots), providing the link between the ubiquitylation pathway and the targeting of gene products to a defined nuclear organelle (35). It needs to be determined what kinds of stimuli cause the ubiquitylation of AIRE under physiological conditions.

Because both a PHD1 mutant (lacking E3 ligase activity) and a PHD2 mutant (devoid of intrinsic transactivation properties (10)) showed distributions similar to that of wild-type AIRE in untreated FLAG-AIRE/NIH3T3 cells, intracellular targeting is independent of the function of PHD domains. This is consistent with a recent report demonstrating that mutated AIRE lacking PHD domains retained at least nuclear entry (36). Upon proteasome inhibition, both FLAG-AIRE/NIH3T3 cells and FLAG-AIRE/mTEC cells transfected with the PHD1 mutant showed similar altered subcellular targeting to that of wild-type AIRE-transfected cells (Fig. 3B and Supplementary Fig. S2), suggesting that auto-ubiquitylation by AIRE itself is not responsible for this action. Supporting this notion, AIRE itself was not polyubiquitylated in the in
vitro ubiquitylation assay when full-sized recombinant AIRE protein was used as an E3 ligase. Therefore, there must be other E3 ligase(s) responsible for AIRE ubiquitylation. Identification of such E3 ligase(s) is required for the elucidation of detailed metabolic and regulatory pathways for the control of AIRE function.

Finally, our results suggest that autoimmunity is controlled in part by multiple regulatory steps affecting the function of transcriptional regulator(s), as exemplified by AIRE. Knowing this, one of the most important questions to be answered is what kind of gene(s) is the target of AIRE. Such putative target gene(s) should maintain self-tolerance by eliminating T cells in the thymus. Supporting this hypothesis, AIRE expression in the thymus was enhanced when negative selection, but not positive selection, was induced (37). Future work will clarify how self-tolerance is physiologically established and maintained in the thymus by studying the link between abnormal function of AIRE at the molecular level and the pathogenetic process of the disease.

**Acknowledgements**

We thank Drs. M. Shono and Y. Kiyosue for help with the confocal microscopic analysis and time-lapse analysis, respectively. We thank M. Ide, K. Awahayashi, S. Matsushita and K. Shinohara for technical assistance. We also thank Dr. S. Ishii for helpful discussion.
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Footnotes

This work was supported in part by Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government (MEXT) (M.M.), by Grant-in-Aid for Scientific Research from the MEXT (S.H. and M.M.), by the Yasuda Research Foundation (S.H.), by the Japan Rheumatism Foundation, by the Yamanouchi Foundation for Research on Metabolic Disorders and by the Uehara Memorial Foundation (M.M.); by the Tampere University Hospital Medical Research Fund, the Emil Aaltonen Foundation, the Finnish Medical Foundation, and the Pirkanmaa Regional Fund of the Finnish Cultural Foundation (J.P.); by the Tampere University Hospital Medical Research Fund, the Finnish Academy, and the Sigrid Juselius Foundation (P.P.); by Grant-in-Aid for Scientific Research (A) and Fund for “Research for the Future” Program from the Japan Society for the Promotion of Science and MEXT (N.S.); by the C.N.R.S. (V.D.).

Abbreviations used in this paper: AIRE, autoimmune regulator; APECED, autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy; PHD, plant homeodomain; NB, nuclear body; PML, promyelocytic leukemia; CBP, CREB-binding protein; Ub, ubiquitin; GFP, green fluorescence protein; mTEC, medullary thymic epithelial cells; DAPI,
4',6-diamidino-2-phenylindole; ALLN, N-acetyl-Leu-Leu-norleucinal; MG132, Z-Leu-Leu-Leu-H; Ab, antibody; mAb, monoclonal Ab; TAP system, tandem affinity purification system; LMB, leptomycin B.
Figure legends

Figure 1. NB formation in HeLa cells stably transfected with the GFP-AIRE fusion gene. Untreated cells (A, left) or cells treated with ALLN, a proteasome inhibitor (A, right), were observed with the aid of fluorescence microscopy. Upon proteasome inhibition, the number and the size of NBs increased in association with the accumulation of many NBs very close to the nucleoli where the DAPI staining is faint (A, right). Green colors represent the fluorescence signals from GFP-AIRE, and DNA staining with DAPI is in blue. Original magnification, x 60. B, Colocalization of GFP-AIRE with endogenous PML was assessed with double immunocytochemistry. Untreated cells were stained with Abs against PML (in red), and subjected to sequential scanning with a confocal microscope. AIRE was not colocalized with PML. Scale bar corresponding to 10 μm is shown in B.

Figure 2. Association of AIRE with the nuclear matrix. A, GFP-AIRE-transfected HeLa cells were extracted sequentially in situ with detergent, DNase I plus ammonium sulfate, and high salt, leaving behind the nuclear matrix. NBs were still present in the nuclear matrix fraction prepared using this treatment. The efficiency of chromatin digestion and removal was assessed by the disappearance of DNA staining with
DAPI, and the presence of nuclear matrix after the treatment was confirmed by staining with anti-lamin A/C mAb. Original magnification, x 40. **B**, Cytoplasmic, chromatin and nuclear matrix proteins were extracted from wild-type HeLa cells (W) or from GFP-AIRE-transfected HeLa cells (A), and GFP-AIRE protein was detected by anti-GFP Ab with Western blot analysis. A significant amount of GFP-AIRE protein was detected in the nuclear matrix fraction obtained from GFP-AIRE-transfected cells (top). Successful fractionation of the cytoplasmic and nuclear matrix proteins was ascertained by anti-IκBα Ab (middle) and anti-lamin A/C mAb (bottom), respectively.

Figure 3. Ubiquitin-dependent subcellular targeting of AIRE. **A**, Subcellular localization of AIRE was determined by immunocytochemical staining of mTEC cells stably transfected with FLAG-tagged AIRE. Upon proteasome inhibition, AIRE in the cytoplasm was decreased, and AIRE predominantly accumulated in the nucleus. Nuclear staining with Hoechst 33258 is in blue. **B**, Proteasome-dependent subcellular targeting of AIRE was investigated in NIH3T3 cells stably transfected with wild-type or PHD-mutant forms of AIRE. Subcellular targeting of AIRE depends on neither E3 ligase activity (PHD1 mutation) nor transactivation properties (PHD2 mutation) in both untreated and MG132-treated cells. (a-f) empty vector; (g-l) wild-type AIRE; (m-r) AIRE
with PHD1 mutation (C299A; PHD1 mt); (s-x) AIRE with PHD2 mutation (C434A; PHD2 mt). C, No colocalization of AIRE with nucleolin in NIH3T3 cells stably transfected with Myc-tagged AIRE after treatment with proteasome inhibitor MG132.

Figure 4. Polyubiquitylation of AIRE is not due to self-ubiquitylation by its E3 ligase activity. A, Myc-tagged AIRE or Myc-tag alone were coexpressed with HA-tagged ubiquitin in COS-7 cells, and the ubiquitylation of AIRE was assessed by immuno-precipitation with anti-Myc mAb followed by detection with anti-HA mAb. The position of Myc-tagged AIRE is indicated by an arrow. B, Immuno-precipitates for AIRE were prepared from the total reaction mixture of in vitro ubiquitylation, which contains full-sized recombinant His-tagged AIRE as E3 ligase, and then subjected to Western blot analysis with anti-Ub Ab. AIRE was not polyubiquitylated, although other substrate(s) are polyubiquitylated in the total reaction mixture (top). Immuno-precipitates for AIRE and total reaction mixture contained similar amount of AIRE as revealed by Western blot analysis with anti-His Ab (bottom).

Figure 5. Preferential colocalization of CBP/p300 with PML bodies rather than with AIRE NBs. In RD cells, CBP/p300 was found in a diffuse
and/or speckled pattern (A, a). When either AIRE or PML was introduced into RD cells, endogenous CBP/p300 was recruited to the AIRE NBs (B, a-c) or to the PML bodies (B, d-f), respectively. When AIRE and PML were introduced simultaneously, AIRE and PML were mostly observed in distinct NB structures (B, g-i). In those cells, CBP/p300 colocalized preferentially with PML bodies (B, j-l) rather than with AIRE NBs (B, m-o). Colocalization of CBP/p300 with PML was observed in a speckled pattern in NB4 cells (A, b). When both AIRE and wild-type PML were transfected, AIRE and PML were mostly observed in distinct NB structures (C, a). In those cells, endogenous CBP/p300 showed colocalization with PML (C, b), but not with AIRE (C, c). Green colors represent the FITC signals, and Texas Red staining is in red. Original magnification, x 60.

**Supplementary Movie 1**

GFP-AIRE/HeLa cells maintained in a 5% CO₂ atmosphere were observed by fluorescence microscopy (Nikon, Tokyo, Japan). Photos were taken every 30 minutes with a digital camera (Hamamatsu Photonics, Hamamatsu, Japan), and images were edited with Adobe Premier software (Adobe Systems, San Jose, CA).

**Supplementary Movie 2**
GFP-AIRE/HeLa cells were treated with 100 M ALLN, and photos were taken as described in the legend to Supplementary Movie 1.

**Supplementary Data**

Figure S1. Association of AIRE with the nuclear matrix. Association of AIRE protein with the nuclear matrix was demonstrated in FLAG-AIRE/mTEC cells (A and B) and Myc-AIRE/NIH3T3 cells (C and D), as described in the legend to Fig. 2.

Figure S2. Ubiquitin-dependent subcellular targeting of AIRE. Subcellular localization of AIRE was determined by calculating the percentages of the cells with nuclear dots before (-) and after (+) treatment with MG132 in FLAG-AIRE/mTEC cells (A) and FLAG-AIRE/NIH3T3 cells (B). As demonstrated in Fig. 3B, PHD1 (C299A) and PHD2 (C434A) mutations did not affect this process. A total of 200 cells with AIRE dots were counted for each group from two independent experiments, and cells having both cytoplasmic and nuclear dots were excluded from the analysis.

Figure S3. *In vivo* expression of AIRE with various molecular masses. Human AIRE expressed in 293T cells with a modified TAP system was subjected to SDS-PAGE, and the protein bands were identified by
MALDI-TOF peptide mass fingerprinting. M: size marker; lane 1: mock transfection with empty vector; lane 2: transfection with human AIRE-expressing vector. The bands excised from the gel and analyzed are marked by arrows with numbers. The bands correspond to: Nos. 1-5, AIRE; No. 6, GRP78 chaperone; No. 7, AIRE and HSP70; No. 8, AIRE; Nos. 9-11, AIRE (degradation form); No. 12, immunoglobulin light chain.
Figure 1  M. Matsumoto
Figure 2 M. Matsumoto
Figure 3  M. Matsumoto

A

|        | Anti-FLAG | Hoechst 33258 | Merge |
|--------|-----------|---------------|-------|
| DMSO   | ![Image](a) | ![Image](b)    | ![Image](c) |
| FLAG-AIRE | ![Image](d) | ![Image](e)    | ![Image](f) |
| MG132  | ![Image](d) | ![Image](e)    | ![Image](f) |
Figure 3  M. Matsumoto

|       | Anti-FLAG | Hoechst 33258 | Merge |
|-------|-----------|---------------|-------|
| **B** |           |               |       |
| **Mock** |     |               |       |
| DMSO | a    | b             | c     |
| MG132 | d    | e             | f     |
| **FLAG-AIRE WT** |     |               |       |
| DMSO | g    | h             | i     |
| MG132 | j    | k             | l     |
| **FLAG-AIRE PHD1 mt** |     |               |       |
| DMSO | m    | n             | o     |
| MG132 | p    | q             | r     |
| **FLAG-AIRE PHD2 mt** |     |               |       |
| DMSO | s    | t             | u     |
| MG132 | v    | w             | x     |
Figure 4 M. Matsumoto

A

HA-Ub:

Ubiquitinilated Myc-AIRE

Myc-AIRE

IP: Anti-Myc

IB: Anti-HA

IB: Anti-Myc

B

Rabbit IgG

Anti-His

Reactions

kDa
200
127
85
41

kDa
209
120
78

IB: Anti-Ub

IB: Anti-His

Ub-proteins

Id-AIRE

IgG
Figure 5  M. Matsumoto

A

B

C
Figure S1  M. Matsumoto

AIRE Lamin A/C DAPI

Untreated

Cytosolic extraction

DNase I + (NH₄)₂SO₄

2M NaCl wash

Cytoplasm Chromatin 2M NaCl Nuclear Matrix

W A W A W A W A

AIRE

I B

Lamin A/C
Figure S1  M. Matsumoto

C

AIRE  Lamin A/C  DAPI

Untreated

Cytosolic extraction

DNase I + (NH4)2SO4

2M NaCl wash

D

|                | Cytoplasm | Chromatin | 2M NaCl | Nuclear Matrix |
|----------------|-----------|-----------|---------|---------------|
| W A            |           |           |         |               |
| AIRE           | W A       | W A       | W A     | W A A         |

Lamin A/C

Figure S1 M. Matsumoto

AIRE  Lamin A/C  DAPI

Untreated

Cytosolic extraction

DNase I + (NH4)2SO4

2M NaCl wash
Subcellular expression of autoimmune regulator (AIRE) is organized in a spatiotemporal manner

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J. Biol. Chem. published online May 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400702200

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