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Extended HSR/CARD domain mediates AIRE binding to DNA

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ABSTRACT

Autoimmune regulator (AIRE) activates the transcription of many genes in an unusual promiscuous and stochastic manner. The mechanism by which AIRE binds to the chromatin and DNA is not fully understood, and the regulatory elements that AIRE target genes possess are not delineated. In the current study, we demonstrate that AIRE activates the expression of transiently transfected luciferase reporters that lack defined promoter regions, as well as intron and poly(A) signal sequences. Our protein-DNA interaction experiments with mutated AIRE reveal that the intact homogeneously staining region/caspase recruitment domain (HSR/CARD) and amino acids R113 and K114 are key elements involved in AIRE binding to DNA.

1. Introduction

Autoimmune regulator (*AIRE*) is a gene that is defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). AIRE is expressed in medullary thymic epithelial cells (mTECs), where it activates a broad range of genes that exhibit tissue-specific expression in peripheral organs and are therefore designated as tissue-specific antigens (TSAs) [1]. In the thymus, promiscuously expressed AIRE-activated TSAs are presented to developing thymocytes, which ensures the clonal deletion of autoreactive T cells [2,3]. In agreement with the tissue-specific expression of AIRE-activated genes, APECED patients and Aire-deficient mice develop circulating organ-specific autoantibodies and autoimmune infiltrations to peripheral tissues [4].

Intracellularly, the AIRE protein resides inside the nucleus, where it exhibits a speckled localization pattern [5]. The nuclear localization signal (NLS) was initially characterized as bipartite, containing two stretches of basic amino acids (aa) at positions 110–114 and 131–133 [6]. Later, only aa 131–133 were confirmed to be important for nuclear import [7].

Although AIRE binding to DNA via its SAND (Sp100, AIRE-1, NucP41/75, DEAF-1) domain [8,9] has been reported, the classical KDWK DNA-binding motif of the SAND domain is changed to KNKA in AIRE, and more recent results did not confirm SAND involvement in DNA binding [10]. The AIRE protein harbors two plant homeodomain (PHD) fingers, of which PHD1 interacts with histone H3 unmethylated at K4 and helps to recruit AIRE to the chromatin [11,12]. In its N-terminus, AIRE has the HSR/CARD domain that is needed for AIRE homomerization and its transcriptional upregulation [13]. Two studies that involved extensive screening of interacting proteins suggested that AIRE induces gene expression via several mechanisms involving transcription, chromatin remodeling, pre-mRNA processing and DNA repair [14,15].

AIRE preferentially activates genes that are tissue specific and characterized by low levels of initial expression [16]. Several studies suggest that AIRE participates in releasing stalled RNA polymerase II into the elongation phase [17,18]. Recent analysis at the single cell level confirmed the stochastic nature of AIRE action and proposed the involvement of DNA organization and epigenetic cues in determination of the target genes [19]. Despite these studies, the transcriptional elements that are needed for AIRE action in the regulation of gene expression are not understood.

In this study, we used a panel of chromatin immunoprecipitation (ChIP) experiments and luciferase activity assays with variety of reporter constructs to elucidate the importance and understand the role of transcriptional and RNA processing elements in the AIRE-mediated upregulation of gene expression. We show that AIRE is able to activate luciferase reporter plasmids without promoter, intron and polyadenylation signal sequences, and we

Abbreviations: AIRE, autoimmune regulator protein; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; ChIP, chromatin immunoprecipitation; HSR/CARD, homogeneously staining region/caspase recruitment domain; mTEC, medullary thymic epithelial cell; SAND, Sp100, AIRE-1, NucP41/75, DEAF-1 domain; PHD, plant homeodomain; qPCR, quantitative real-time PCR.

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demonstrate that the AIRE HSR/CARD domain, as well as aa R113 and K114, are important for AIRE binding to DNA.

2. Materials and methods

2.1. Plasmids

The following full-length AIRE expression constructs were used: pcAIRE [20], pcAIRE-D312A [21], pcAIRE-K243/245Q [22], and pcAIRE-L28P [23]. pcAIRE-R15C, A21V, L29P, V80L, K83E, and L93R were generated by PCR-based mutagenesis and cloned into the EcoRI/HindIII sites of pcDNA3.1 B(−)Myc/His. To generate pcHSR/CARD (aa 1–109) and pcHSR/CARD+ (aa 1–143), the corresponding regions were PCR amplified from pcAIRE and cloned into the NotI/EcoRI sites of pcDNA3.1 B(−)Myc/His. pcHSR/CARD+R113A/K114A was generated by PCR-based site-directed mutagenesis. pcDNA3.1B(−)Myc/His (Invitrogen), pGL3-Basic (Promega), pBL-KS (a gift from prof. K. Saksela, University of Tampere, Finland) and pd2EYFP-N1 (Clontech) were used as the controls. The lack of promoter sequences in pGL3-Basic and pBL-KS plasmids was confirmed by GenBank and Eukaryotic Promoter Database searches.

The luciferase reporter pBL-INV (pBL-IVL in this study) containing promoter area of involucrin (3737 nt) has been described previously [24]. IVL-pr (nt −259/−1), IVL-pr-in (nt −259/+1235), IVL-in (nt −27/+1235), INS-pr (nt −326/+30), INS-pr-in (nt −326/+238) and INS-in (nt −5/+238) were PCR amplified from pBL-IVL or human genomic DNA and cloned into HindIII/BamHI sites of pBL-KS vector. The AdML intron was amplified from the sequence of the AdML splicing substrate [25] and cloned into the BamHI site of IVL-pr or INS-pr. Luc-SL, Luc-TL and Luc-MS2 were gifts from B. Marzluff and were described previously [26]. To generate GST-HSR/CARD (aa 1–109) and GST-HSR/CARD-L28P, the corresponding regions were PCR amplified from pcAIRE and pcAIRE-L28P, respectively, and cloned into the EcoRI/XhoI sites of pGEX-1λT vector. GST-HSR/CARD+ (aa 1–138) (previously named as GST-AIRE-(1–138)) was previously described [13]. pcDNA-p50 and pcDNA-FLAG-p65 were a gift from prof. E. Kalkhoven from Utrecht University. The 3xNF-κBLuc reporter plasmid was a gift from prof. K. Saksela.

All generated constructs were verified by sequencing. Cloning and mutagenesis primers are available upon request.

2.2. Cell culture and transfections

The human embryonic kidney cell line HEK293 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (PAA). All transfections were performed with TurboFect *in vitro* Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol.

2.3. Luciferase reporter assay and quantitative PCR

Cells were seeded at 20–30% confluency in 24-well plates 24 h before the transfection. A total of 1 μg of plasmid (with a 1/10 expression/reporter plasmid ratio) per well was used. Cells were harvested 48 h later, and luciferase (Luc) activity was measured using a Luciferase Assay System (Promega) according to the manufacturer's instructions. The transfections were performed in duplicate, and experiments were repeated at least two times. Luminescence was counted with a Wallac 1420 Victor Multilabel/Plate Reader (Perkin Elmer). RNA was purified with TRIzol-chloroform extraction, reverse transcribed with SuperScript III (Life Technologies) and treated with TURBO DNaseI (Thermo Fisher Scientific). The qPCR was performed in triplicate on ViiA™ 7 PCR System using primers F-CCTTACTGTGAGTCTGGTTGACA and R-CTTTCTTTATGTTTTTGGCGTCTTCCAT for IVL spliced mRNA detection and primer pairs F-TTGGCTATGCTGCACAGAGG, R-GAGCTGGAAGGTTGCTGAGA, F-TCTAGGGCGCAGTAGTCCAG, R-TACTGGAAAGACCGCGAAGA inside *IVL* and adenoviral intron, respectively. Relative gene expression levels were calculated using the comparative Ct (ΔΔCt) method (according to Applied Biosystems) and *HPRT* was used as house-keeping gene for normalization.

2.4. Chromatin immunoprecipitation

A total of 5×10^6 transfected HEK293 cells were used in a single chromatin immunoprecipitation (ChIP) reaction with the following antibodies: mouse monoclonal anti-c-Myc (ab32, Abcam), rabbit polyclonal anti-Histone H3 (ab1791, Abcam) and rabbit IgG, as a negative control (Chemicon International). ChIP was performed according to the previously described protocol [21]. Purified DNA from ChIP samples were analyzed by quantitative PCR in triplicate using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and a ViiA 7 Real-Time PCR System (Life Technologies). The results were analyzed using the *Ct* method, as previously described [21].

2.5. The expression and purification of GST fusion proteins and electrophoretic mobility shift assays

GST fusion proteins were expressed and purified as previously described [24].

Electrophoretic mobility shift assay (EMSA) was performed according to a previously described protocol [27]. The proteins were probed with a P^{32}-labeled duplex oligonucleotide containing the FLAG tag coding sequence: 5′-AATTGAATTCGATTACAAGGACGACGATGACAAGTAGCTTAAGTTAA-3′ without any known recognition elements for transcription factor binding. EMSA in agarose gel was performed as previously described [28] using pcDNA3 (circular or linearized with HindIII) as a probe.

2.6. Immunofluorescence

The immunofluorescence protocol described in Ref. [22] with mouse monoclonal anti-Myc (ab32, Abcam) and anti-mouse IgG conjugated with Alexa-488 (A11029, Invitrogen) antibodies has been used. The images were captured with the LSM5 DUO confocal microscope (Zeiss) and processed with LSM Image Browser software (Zeiss).

3. Results

3.1. AIRE can activate promoterless reporter plasmids

We and others have previously reported that AIRE can activate the transcription of many genes in genomic DNA context and can induce the expression of reporter genes from various promoters inserted into plasmids [2,5]. Two marker genes, involucrin (*IVL*) and insulin (*INS*) have been characterized as AIRE-activated genes in primary mTECs and HEK293 cells as well as when cloned into plasmids [2,21]. To identify the transcriptional elements that are required for AIRE-induced activation, we performed Luc reporter assays using constructs containing the promoters of *IVL* (IVL-pr) and *INS* (INS-pr), and used pBL-KS and pGL3 luciferase vectors as negative controls (Fig. 1A). Unexpectedly, we found that in addition to the IVL-pr and INS-pr constructs, AIRE was able to activate Luc expression from pGL3 and pBL-KS plasmids without any promoter elements. Although the Luc activity in cells transfected with control

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