Deaf1 isoforms control the expression of genes encoding peripheral tissue antigens in the pancreatic lymph nodes during type 1 diabetes

Linda Yip¹, Leon Su¹, Deqiao Sheng¹, Pearl Chang¹, Mark Atkinson², Margaret Czesak³, Paul R. Albert³, Ai-Ris Collier⁴, Shannon J. Turley⁴, C. Garrison Fathman¹, and Rémi J. Creusot¹

¹ Department of Medicine, Division of Immunology and Rheumatology, Stanford University School of Medicine, Stanford, California, 94305, USA
² Department of Pathology, University of Florida, Gainesville, FL, 32610, USA
³ Ottawa Health Research Institute (Neuroscience) and Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada
⁴ Department of Cancer Immunology and AIDS, Dana Farber Cancer Institute, Boston, Massachusetts, 02115, USA

Abstract

Type 1 diabetes (T1D) may result from a breakdown in peripheral tolerance that is partially controlled by peripheral tissue antigen (PTA) expression in lymph nodes. Here we show that the transcriptional regulator deformed epidermal autoregulatory factor 1 (Deaf1) controls PTA gene expression in the pancreatic lymph nodes (PLN). The expression of canonical Deaf1 was reduced, while that of an alternatively spliced variant was increased during the onset of destructive insulinitis in the PLN of NOD mice. An equivalent variant Deaf1 isoform was identified in the PLN of T1D patients. Both NOD and human Deaf1 variant isoforms suppressed PTA expression by inhibiting the transcriptional activity of canonical Deaf1. Reduced PTA expression resulting from the alternative splicing of Deaf1 may contribute to T1D pathogenesis.
INTRODUCTION

Type 1 diabetes (T1D) results from the autoimmune destruction of pancreatic β-cells by self-reactive T cells in genetically susceptible individuals due to a breakdown in central and/or peripheral tolerance. Central tolerance is enforced by medullary thymic epithelial cells (mTECs) that ectopically express a range of peripheral tissue antigens (PTAs) under the transcriptional control of the autoimmune regulator, Aire. Most maturing thymocytes that express T cell receptors (TCRs) specific for these PTAs are deleted, but some self-reactive T cells escape to the periphery where additional tolerance mechanisms are in place to protect tissues from autoimmune attack. In addition to regulatory T cells, peripheral tolerance can be mediated by lymph node stromal cells (LNSCs) and extrathymic Aire-expressing cells (eTACs), which present PTA peptides on MHC molecules and engage autoreactive PTA-specific T cells, ultimately leading to their clonal deletion. The mechanism(s) that controls PTA expression in secondary lymphoid tissues are not completely understood.

We previously compared the gene expression in tissues from non-obese diabetic (NOD) mice, a model of T1D, to that of NOD.B10 mice, a congenic strain that does not develop disease. We found that the expression of the gene encoding the transcriptional regulator deformed epidermal autoregulatory factor 1 (Deaf1) changed in parallel with the expression of genes encoding a number of islet-specific tissue antigens including insulin 1 (Ins1), insulin 2 (Ins2), glucagon (Gcg), pancreatitis-associated protein (Pap), pancreatic polypeptide (Ppy), and regenerating islet-derived alpha and gamma (Reg3a and Reg3g) in the pancreatic lymph nodes (PLN). The expression of Deaf1 and genes encoding these islet-specific antigens was significantly downregulated in the PLN of NOD mice at the age of 12 weeks, a time coincident with the onset of destructive insulitis.

In addition, we demonstrated that Deaf1 regulates the expression of certain PTA genes in lymph node stromal elements (LNSE). We found that Deaf1 can be processed into at least two isoforms in the PLN of NOD mice: a canonical Deaf1 isoform that can translocate to the nucleus and is required for PTA expression, and a splice variant that is expressed in the cytoplasm and inhibits the transcriptional activity of canonical Deaf1. Remarkably, expression of a similar alternatively spliced Deaf1 isoform was significantly increased in the PLN of T1D patients.

Results

Deaf1 isoforms and PTA expression in the NOD PLN

Microarray analysis demonstrated that the expression of various pancreatic genes and Deaf1 change in parallel over time in the PLN of NOD mice (Fig. 1a,b). Two probes (probe 1 and 2) that bind to Deaf1 were present on the microarray, but only probe 1 revealed an expression profile similar to that of the pancreatic PTA genes (Fig. 1a). Probe 1 hybridizes to a Deaf1 region spanning exon 6 and 7, while probe 2 hybridizes to a region within exon 11 (Fig. 1c). The discordant expression profiles revealed by the two probes suggested that more than one Deaf1 isoform might exist in the PLN, and that the relative expression of the two isoforms might change with age.
PCR cloning and sequence analysis of *Deaf1* mRNA in PLN tissue identified the canonical mRNA (*DF1*; Genbank Accession FJ377319) as well as an alternatively spliced transcript containing an insertion between exons 6 and 7 (*DF1-VAR1*; Genbank Accession FJ377318; Fig. 1c) that results in disruption of the binding site of probe 1. Thus, changes in the expression of these two *Deaf1* isoforms could explain the discordant patterns of expression revealed by the two probes (Fig. 1b).

The microarray data (Fig. 1a) used a pool of PLN mRNA from 1.5 and 20 week old NOD.B10 mice as controls. Next, we quantified *DF1* and *DF1-VAR1* expression in NOD and NOD.B10 mice at various ages by quantitative PCR (QPCR) (Fig. 2 and Supplementary Fig. 1 and 2). The Taqman probe for *DF1* spans exons 6 and 7, at the intronic insertion site, and the *DF1-VAR1* primer and probes were designed to hybridize within the intronic insertion (Supplementary Fig. 1).

*DF1* and *DF1-VAR1* expression did not differ between the PLN of NOD and NOD.B10 mice at 4 weeks of age (Fig. 2a, b). However, at 12 weeks, *DF1* mRNA expression was downregulated and *DF1-VAR1* expression was upregulated specifically in the NOD PLN. In contrast, *Gapdh* mRNA expression did not differ between 12-week NOD vs. NOD.B10 PLN (Fig. 2c). The changes in *DF1* and *DF1-VAR1* gene expression appear specific to the PLN, as no significant change was observed in spleen tissues of 12-week old NOD and NOD.B10 (Fig. 2d).

**Characterization of Deaf1 isoforms**

Canonical *DF1* contains an N-terminal alanine-rich region, the SAND and ZF-MYND domains, a helix-loop-helix (HLH) domain, and nuclear localization (NLS) and nuclear export sequences (NES). The *DF1-VAR1* transcript contains part of intron 7, which introduces a premature stop codon that results in a truncated protein lacking part of the NLS as well as the HLH, NES and ZF-MYND domains. *DF1-VAR1* also contains a 42 bp deletion in the alanine-rich region (Fig. 1c).

*DF1* is ~70 kDa and localized in the nucleus of HEK 293 cells (Fig. 3a, b). *DF1-VAR1* is ~50 kDa and was distributed throughout the cytoplasm (Fig. 3a, b). Interestingly, transient co-expression of *DF1* with *DF1-VAR1* resulted in nuclear localization of some *DF1-VAR1*, as revealed by immunofluorescence (Fig. 3b). This finding was confirmed by immunoblot experiments (Fig. 3c). Co-immunoprecipitation studies using Flag-tagged *DF1* and V5 epitope tagged *DF1-VAR1* demonstrate that *DF1* and *DF1-VAR1* can interact (Fig. 3d). These data suggest that the *DF1* and *DF1-VAR1* form hetero-dimeric complexes that can shuttle *DF1-VAR1* into the nucleus using the NLS domain of *DF1*.

The transcriptional activity of *DF1* and *DF1-VAR1* was examined using a 26 bp *Deaf1*-response element-luciferase reporter plasmid. When transiently transfected into HEK 293 cells, *DF1-EGFP* induced greater luciferase reporter activity than *DF1-VAR1-EGFP* (Fig. 3e). The lower transcriptional activity of *DF1-VAR1* may be due to its cytoplasmic localization and reduced ability to translocate to the nucleus (Fig. 3b). Co-transfection of *DF1-VAR1* and *DF1* resulted in substantially lower transcriptional activity than that of equivalent amounts of *DF1* alone (Fig. 3f).
Analysis of Deaf1-deficient BALB/c mice

To examine the genes that are regulated by Deaf1 in the PLN, whole genome microarrays were performed on Deaf1-deficient BALB/c PLN tissues. Approximately 300 genes were upregulated and 300 were downregulated by ~2.5 fold in Deaf1-KO mice compared to BALB/c controls (Fig. 4a; Supplementary Table 1 and 2). Interestingly, among the top 30 genes whose expression was downregulated in Deaf1-KO PLN, almost three-quarters were considered potential PTAs with expression limited to ≤ 5 tissues; in contrast less than a quarter of the middle 30 genes, whose expression was not changed in the Deaf1-KO mice, were expressed in ≤ 5 tissues (Supplementary Table 3). The genes regulated by Deaf1 in the PLN did not appear to significantly overlap with genes regulated by Aire in eTACs (Supplementary Fig. 3).

Two Aire-regulated genes, Ambp and Fgb, were found among the top 20 downregulated genes in the PLN of Deaf1-KO mice. Reg3g and Ppy, which encode pancreas-specific antigens, are also regulated by Aire in the thymus1. To accurately measure the expression of Reg3g, Ppy, Ambp and Fgb in the PLN of Deaf1-KO and control mice, SYBR green QPCR assays were used. The specificity and amplification efficiencies of each assay were confirmed by RT-PCR, melting curve analysis and standard curves (Supplementary Fig. 4). The expression of Reg3g, Ppy, Ambp and Fgb was significantly lower in the PLN of Deaf1-KO mice than WT controls (Fig. 4b). Interestingly, the expression of these same genes was upregulated in the thymus of Deaf1-KO mice, possibly due to increased Aire mRNA expression in the thymus of Deaf1-KO mice (Fig. 4c); Aire mRNA quantities were not different in the PLN of Deaf1-KO and control mice (Fig. 4b). Surprisingly, Ins2 mRNA in the PLN of Deaf1-KO and BALB/c control mice was found to be 30 to 1700-fold lower than that of NOD and NOD.B10 mice (Supplementary Fig. 5), and thus was not detected in our microarray analysis.

To determine if 30-week old Deaf1-KO mice exhibited signs of autoimmunity, we looked for lymphocyte infiltration in various tissues and for the presence of autoantibodies. Lymphocyte infiltration was not detected in the pancreas, salivary glands, thyroid, gut or kidney (data not shown). The serum of Deaf1-KO mice reacted with certain proteins (~15–25 kDa) in whole eye lysate (Fig. 4d), but not with lysates of any other tissues examined (brain, salivary gland, heart, lung, liver, kidney, pancreas, spleen, and gut). The autoantibodies in the Deaf1-KO serum reacted with the outer segment layer of the retina, similar to autoantibodies in Aire-KO mice (Fig. 4e).

Influence of Deaf1 isoforms on PTA expression

Next we silenced the expression of Deaf1 in NIH 3T3 cells, which express endogenous Deaf1, Ambp and Fgb, using siRNA to examine Deaf1-mediated regulation of these PTAs. Deaf1 siRNA significantly inhibited the expression of DF1-Flag as well as endogenous Deaf1 in NIH 3T3 cells, demonstrating the specificity of the Deaf1 siRNA (Fig. 5a,b). Deaf1 siRNA, compared to nonsense siRNA, also resulted in a significant suppression of Ambp and Fgb expression (Fig. 5c). However, over-expression of DF1 in NIH 3T3 cells did not upregulate the expression of Fgb, but only slightly increased the expression of Ambp (Supplementary Fig. 6). These data indicate that, while a decrease in DF1 expression can...
reduce PTA gene expression, an increase in DF1 expression alone may not be sufficient to enhance PTA gene expression. This conclusion is supported by data in the NOD PLN where decreased DF1 and increased DF1-VAR1 expression in 12-week old NOD mice strongly correlated with significantly reduced PTA gene expression (Fig. 2 and 6a), whereas increased DF1 expression detected in 20 week NOD PLN did not correlate with increased PTA gene expression (Fig. 6a).

Next we sought to identify the cell type(s) in which changes in DF1-VAR1 expression occur in the 12-week NOD PLN. We found that DF1-VAR1 expression was approximately 7-fold and 10-fold higher in LNSEs from 12 week old NOD PLN than in T cells and B cells from the same mice (Fig. 6b). Silencing of Deaf1 in immortalized lymph node CD45− cells from pooled lymph nodes of BALB/c mice resulted in a decrease in Ambp and Fgb mRNA expression (Fig. 6c,d). Similarly, overexpression of DF1-VAR1 in these cells reduced Ambp and Fgb expression (Fig. 6e). Although overexpression of DF1-VAR1 did not alter the expression of DF1 (Fig. 6e), we propose that it interfered with the transcriptional activity of DF1 by interacting with and retaining it in the cytoplasm. Supporting this hypothesis, immunoblot experiments performed with nuclear and cytoplasmic extracts of HEK 293 cells transfected with DF1-Flag and/or DF1-VAR1-EGFP showed that the amount of cytoplasmic DF1 was higher and the amount of nuclear DF1 was lower in cells transfected with both DF1-VAR1 and DF1 compared to cells transfected with DF1 alone (Fig. 6f). These data suggest that DF1-VAR1 can reduce PTA gene expression by interacting with and retaining DF1 in the cytoplasm.

Alternatively spliced human Deaf1 variant

To examine if an alternatively spliced variant of DEAF1 is expressed in human PLN, RT-PCR was performed with human PLN cDNA as a template and primers that span the start and stop codons of the human DEAF1 gene (Supplementary Table 4). These primers amplified the canonical human DEAF1 transcript (Hu-DF1; Genbank Accession No. BC053322), as well as an alternatively spliced variant (Hu-DF1-VAR; Genbank Accession No. FJ985253) that lacks exons 5 and 7 and the first nucleotide of exon 6. This variant transcript also contains an intronic 42 bp insertion between exons 10 and 11 that is expressed “in-frame” from the start to stop codons, and contains intact SAND and MYND domains, but lacks the NLS domain encoded by exon 7 (Fig. 7a).

Characterization of the Hu-DF1-VAR isoform revealed a number of similarities with the mouse DF1-VAR1 isoform. Hu-DF1-VAR was expressed predominantly in the cytoplasm (Fig. 7b) and did not mediate transcriptional activation of the 26 bp Deaf1 response element (Fig. 7c). This variant interacted with canonical Hu-DF1 and altered its distribution within the cell. When expressed alone, Hu-DF1 localized almost exclusively in the nucleus (Fig. 7b, d), but when co-expressed with Hu-DF1-VAR its nuclear and cytoplasmic expression decreased by ~3-fold and increased by ~4-fold, respectively. On the other hand, co-expression of the two isoforms enhanced the nuclear localization of Hu-DF1-VAR (Fig. 7e).

To examine if the alternatively spliced isoform of DEAF1 is upregulated in the PLN during T1D, QPCR was performed using primers that span the two deletion sites of the Hu-DF1-VAR transcript (Table 1 and Supplementary Fig. 7). Expression of Hu-DF1-VAR was ~20
fold higher in the PLN of T1D patients than in healthy controls (Fig. 7f). This finding strongly correlated with the lack of INS expression observed in the PLN of all T1D patients studied (Table 1). INS expression was detected in 4 of 5 of the control PLN, and in the spleen of all samples. The spleen samples expressed either no Hu-DF1-VAR or Hu-DF1-VAR amounts comparable to those detected in the control PLN (data not shown). Together, these data suggest that during the progression of human T1D, DEAF1 is alternatively spliced to produce a nonfunctional variant similar to mouse DF1-VAR1. The Hu-DF1-VAR isoform interacts with and inhibits the transcriptional activity of canonical Hu-DF1 by retaining it in the cytoplasm.

Discussion

T1D develops from a breakdown in central or peripheral tolerance that is partially controlled by PTA expression in the thymus and lymph nodes, respectively. Here we demonstrate that Deaf1 plays a role in regulating PTA gene expression in peripheral lymphoid tissues. We found that the transcriptional activity of Deaf1 can be inhibited by alternatively spliced variants of Deaf1, and that the expression of these splice variants was significantly higher in the PLN of T1D patients and 12 week old NOD mice than in controls. These findings suggest that alternative splicing of Deaf1 could play a key role in the pathogenesis of T1D and NOD disease.

The age of 12 weeks is pivotal in the progression of NOD disease, as infiltrative insulitis and β-cell destruction begins at this age. Peripheral tolerance to certain PTAs is facilitated in the lymph nodes through the ectopic expression of PTA genes in stromal cells, and defects in peripheral tolerance may induce infiltrative insulitis. The expression of genes encoding various pancreatic PTAs is diminished in the PLN of NOD mice at 12 weeks of age. This coincides with a downregulation of DF1 and upregulation of DF1-VAR1 gene expression. Since DF1-VAR1 inhibits PTA gene expression and interferes with the transcriptional activity of DF1, a change in Deaf1 isoform expression may result in decreased PTA expression in 12 week old NOD PLN and reduced tolerance to these antigens.

Due to the obvious practical difficulties in obtaining human PLN tissues from pre-diabetic individuals as well as those at disease onset, we could not assess the expression of Hu-DF1 and Hu-DF1-VAR throughout all stages in the natural history of T1D. In the one pre-diabetic PLN sample we obtained, Hu-DF1-VAR expression was higher than in the PLN of all the healthy control samples examined. Interestingly, the increased expression of Hu-DF1-VAR and absence of INS in the PLN appeared to be maintained well after the onset of T1D.

Increased expression of the Deaf1 variant can inhibit the transcriptional activity of canonical Deaf1 by forming hetero-dimeric complexes that retain the canonical isoform in the cytoplasm. Both DF1-VAR1 and Hu-DF1-VAR lack the NLS domain that is required for nuclear localization and activation of gene transcription, and both induced minimal transcriptional activity of the 26-bp Deaf1 response element compared to that induced by DF1 and Hu-DF1. The canonical isoform of Deaf1 is structurally similar to Aire, which also contains a SAND domain and can induce PTA gene expression by interacting with modified

Yip et al. Page 6

Nat Immunol. Author manuscript; available in PMC 2010 March 01.
and unmodified histone H3 via its PHD-ZF domain. We suggest that Deaf1, rather than Aire, plays a role in controlling PTA expression in the PLN. Deaf1 may regulate PTA expression in a manner analogous to Aire since both proteins contain similar functional domains: Deaf1 contains a C-terminal ZF-MYND domain that is structurally similar to the PHD-ZF, as well as a SAND domain that functions as a DNA binding domain for chromatin-dependent transcription and a site for protein-protein interaction. However, the effect of Deaf1 function may be cell-dependent. For example, Deaf1 represses and enhances promoter activity of the 5-HT1A receptor in presynaptic and postsynaptic neurons, respectively. In addition, some genes were upregulated while others were downregulated in Deaf1-KO mice, suggesting that Deaf1 may interact with cell-specific regulatory factors to either stimulate or suppress gene transcription. The requirement for additional factors may explain why overexpression of DF1 did not significantly increase PTA gene expression in NIH 3T3 cells, and why changes in PTA gene expression in PLN tissue did not necessarily correlate with increased DF1 expression.

BALB/c mice, in which Deaf1 was knocked out, did not manifest an obvious autoimmune phenotype. However, like Aire-KO mice, the serum of Deaf1-KO mice contained autoantibodies that were immunoreactive against proteins in the retina of the eye. Interestingly, our microarray data showed that the most downregulated gene in the PLN of Deaf1-KO mice is dopachrome tautomerase (Dct), which encodes a protein that is expressed exclusively in the retina. In addition, three other genes among the top 30 genes that were downregulated in the PLN of Deaf1 mice (150016O10Rik, Si, and Sgne1) are expressed in the retina. The control of retinal antigen expression by Deaf1 may be of particular significance, as reduced antigen presentation in eye-draining lymph nodes prevents autoreactive T cell deletion and contributes to the development of retinal autoimmunity.

Variations in thymic PTA gene expression can influence susceptibility to autoimmune disease. The expression of genes encoding certain PTAs was reduced in the PLN of Deaf1-KO mice, while surprisingly, the expression the same genes and of Aire was increased in the thymus of the Deaf1-KO mice. This suggests the possibility that enhanced central tolerance mechanisms, mediated by increased Aire-regulated expression of PTAs in the thymus, may result in the lack of overt autoimmunity in the Deaf1-KO mice. Strain-dependent variations in the thymic expression of genes encoding certain PTAs, such as the uveitogenic retinal antigen interphotoreceptor retinoid binding promoter (IRBP), have been observed. Previous studies have also shown that the severity of the autoimmune phenotype of Aire-knockout mice depends on the genetic background. Aire-deficient mice developed severe exocrine pancreatitis in NOD but not in C57BL/6 or BALB/c backgrounds, while autoimmune gastritis and auto-antibodies against Mucin-6 developed in Aire-deficient mice in NOD and BALB/c backgrounds, but not in the C57BL/6 background. The phenotype of the Deaf1-KO is also strain-specific. In the C57BL/6 background, Deaf1-KO mice suffered from various developmental defects that were not observed in BALB/c Deaf1-KO mice.

Ambp, Fgb, Ppy and Reg3g were downregulated in the PLN of Deaf1-KO mice, and we selected these genes for further study based on their regulation by Aire in the thymus and reduced expression in the PLN of 12 week old NOD mice. In mTECs and LNSCs, PTAs are regulated by Aire and other transcription factors.
expressed in low amounts. For example, PTA expression in lymph nodes was assessed using the iFABP-tOVA transgenic mouse model, where OVA (ovalbumin) represents a self-antigen expressed under the control of the fatty acid binding protein (FABP) promoter. OVA mRNA expression was detected in lymph nodes by RT-PCR, but OVA protein was not detected by immunoblotting. Similarly, PTA expression is low in the thymus, as only a small subset of mTECs ectopically express Aire and PTAs.

In humans, INS was not detected in the PLN of T1D patients, but was expressed in the PLN of healthy individuals and spleens of both control and T1D samples. The lack of INS expression correlated well with the high expression of Hu-DF1-VAR expressed in the PLN of T1D patients. In 12-week old NOD PLN, Ins2 gene expression was also reduced, but we could not detect a difference in Ins2 mRNA expression in the PLN of Deaf1-KO mice compared to BALB/c control mice. This may be due to the significantly lower expression of Ins2 mRNA in the PLN of Deaf1-KO and BALB/c control mice (30 to 1700-fold lower) compared to that of NOD and NOD.B10 mice.

Members of the Reg family have been described as autoantigens in T1D, and the inflammation-induced upregulation of Reg expression in islets was suggested to contribute to the premature onset of diabetes. We showed that Reg3g expression was downregulated in the PLN of Deaf1-KO mice and 12-week old NOD mice, but were unable to determine if Deaf1 directly regulated Reg3g expression since the NIH 3T3 and LN CD45− cell lines that we used for the siRNA experiments did not express measurable amounts of Reg3g. A loss of endogenous PTA gene expression has been shown to occur in cultured mTEC24 and LNSC lines (unpublished data). Thus, future experiments involving monoclonal antibodies to DF1-VAR1 and/or in situ hybridization studies of primary lymph node stromal elements derived from the PLN of NOD and NOD.B10 mice may be necessary to identify the actual stromal cell that expresses the variant Deaf1 isoforms and to determine the direct role of Deaf1 in the expression of PTA genes.

While the expression of Ambp, Fgb, Ppy, and Reg3g is regulated by Aire in the thymus, the expression of these genes in the PLN is more likely regulated by Deaf1. Aire expression was not altered in the PLN of Deaf1-KO mice or NOD mice, and most of the PTAs regulated by Aire in eTACs appear to be distinct from those regulated by Deaf1 in the PLN.

Our study suggests that Deaf1 promotes the ectopic expression of genes encoding PTAs in the PLN, and PTA expression in LNSCs can mediate peripheral tolerance. Thus, fine-tuning of peripheral tolerance, at least in the PLN, may occur through alternative splicing of Deaf1. Alternative splicing is a mechanism that is often used to control immune responses. However, variations in splicing can impair immune function and contribute to various autoimmune diseases. It is unclear how splicing of Deaf1 is controlled, but inflammation of the NOD PLN may be involved since various inflammatory cytokines have been shown to induce alternative splicing. Differences in Deaf1 splicing in NOD vs. NOD.B10 mice may also be due to other genes within the Idd1 susceptibility region, a MHC congenic interval that distinguishes the two strains. Allelic variation of such genes or differences in their regulatory regions may influence their expression or function, which may be associated with splicing events.
We propose that during the progression of T1D and NOD disease, alternative splicing of Deaf1 occurs in the PLN. The alternatively spliced Deaf1 variant interacts with and retains the canonical isoform in the cytoplasm and inhibits PTA gene expression. Decreased expression of pancreatic antigens in the PLN may impair peripheral tolerance and lead to the survival of autoreactive T cells specific for these antigens. Thus, we suggest that differences in the expression of Deaf1 isoforms in the PLN of humans with T1D and NOD mice may contribute to the development of this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank H. Iwai and C. Taylor for their technical assistance, J. Visvader (The Walter and Eliza Hall Institute of Medical Research) for providing Deaf1-KO mice, and the JDRF nPOD for providing human PLN and spleen samples. This work was funded by NIH grants U01 DK078123-03, U19- DK 61934 and U19- AI050864 (to C.G.F.), and the Canadian Institutes of Health Research (CIHR; to P.R.A). Linda Yip was supported by the American Diabetes Association Mentor-based Postdoctoral Fellowship; Margaret Czesak received a CIHR Studentship.

References

1. Anderson MS, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science. 2002; 298:1395–1401. [PubMed: 12376594]
2. Lee JW, et al. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. Nat Immunol. 2007; 8:181–190. [PubMed: 17195844]
3. Gardner JM, et al. Deletional tolerance mediated by extrathymic Aire-expressing cells. Science. 2008; 321:843–847. [PubMed: 18687966]
4. Nichols LA, et al. Deletional self-tolerance to a melanocyte/melanoma antigen derived from tyrosinase is mediated by a radio-resistant cell in peripheral and mesenteric lymph nodes. J Immunol. 2007; 179:993–1003. [PubMed: 17617591]
5. Kodama K, et al. Tissue- and age-specific changes in gene expression during disease induction and progression in NOD mice. Clin Immunol. 2008; 129:195–201. [PubMed: 18801706]
6. Lemonde S, et al. Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci. 2003; 23:8788–8799. [PubMed: 14507979]
7. Adorini L, Gregori S, Harrison LC. Understanding autoimmune diabetes: insights from mouse models. Trends Mol Med. 2002; 8:31–38. [PubMed: 11796264]
8. Huggenvik JI, et al. Characterization of a nuclear deformed epidermal autoregulatory factor-1 (DEAF-1)-related (NUDR) transcriptional regulator protein. Mol Endocrinol. 1998; 12:1619–1639. [PubMed: 9773984]
9. Koh AS, et al. Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. Proc Natl Acad Sci U S A. 2008; 105:15878–15883. [PubMed: 18840680]
10. Org T, et al. The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression. EMBO Rep. 2008; 9:370–376. [PubMed: 18292755]
11. Jensik PJ, Huggenvik JI, Collard MW. Identification of a nuclear export signal and protein interaction domains in deformed epidermal autoregulatory factor-1 (DEAF-1). J Biol Chem. 2004; 279:32692–32699. [PubMed: 15161925]
12. Bottomley MJ, et al. The SAND domain structure defines a novel DNA-binding fold in transcriptional regulation. Nat Struct Biol. 2001; 8:626–633. [PubMed: 11427895]
13. Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR. Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci. 2006; 26:1864–1871. [PubMed: 16467535]

14. Lambe T, et al. Limited peripheral T cell anergy predisposes to retinal autoimmunity. J Immunol. 2007; 178:4276–4283. [PubMed: 17371984]

15. Bennett ST, et al. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. Nat Genet. 1995; 9:284–292. [PubMed: 7773291]

16. Egwuagu CE, Charukamnoetkanok P, Gery I. Thymic expression of autoantigens correlates with resistance to autoimmune disease. J Immunol. 1997; 159:3109–3112. [PubMed: 9317106]

17. Avichezer D, et al. An immunologically privileged retinal antigen elicits tolerance: major role for central selection mechanisms. J Exp Med. 2003; 198:1665–1676. [PubMed: 14657219]

18. Venanzi ES, Melamed R, Mathis D, Benoist C. The variable immunological self: genetic variation and nongenetic noise in Aire-regulated transcription. Proc Natl Acad Sci U S A. 2008; 105:15860–15865. [PubMed: 18838677]

19. Gavanescu I, Kessler B, Ploegh H, Benoist C, Mathis D. Loss of Aire-dependent thymic expression of a peripheral tissue antigen renders it a target of autoimmunity. Proc Natl Acad Sci U S A. 2007; 104:4583–4587. [PubMed: 17360567]

20. Hahm K, et al. Defective neural tube closure and anteroposterior patterning in mice lacking the LIM protein LMO4 or its interacting partner Deaf-1. Mol Cell Biol. 2004; 24:2074–2082. [PubMed: 14966286]

21. Derbinski J, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. J Exp Med. 2005; 202:33–45. [PubMed: 15983066]

22. Derbinski J, Schulte A, Kyewski B, Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat Immunol. 2001; 2:1032–1039. [PubMed: 11600886]

23. Planas R, et al. Reg (regenerating) gene overexpression in islets from non-obese diabetic mice with accelerated diabetes: role of IFNbeta. Diabetologia. 2006; 49:2379–2387. [PubMed: 16900387]

24. Kasai M, Kropshofer H, Vogt AB, Kominami E, Mizuochi T. CLIP-derived self peptides bound to MHC class II molecules of medullary thymic epithelial cells differ from those of cortical thymic epithelial cells in their diversity, length, and C-terminal processing. Eur J Immunol. 2000; 30:3542–3551. [PubMed: 11169395]

25. Lynch KW. Consequences of regulated pre-mRNA splicing in the immune system. Nat Rev Immunol. 2004; 4:931–940. [PubMed: 15573128]

26. Eissa NT, et al. Alternative splicing of human inducible nitric-oxide synthase mRNA. tissue-specific regulation and induction by cytokines. J Biol Chem. 1996; 271:27184–27187. [PubMed: 8900212]

27. Gratchev A, et al. Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. Scand J Immunol. 2001; 53:386–392. [PubMed: 11285119]

28. Borsi L, Castellani P, Risso AM, Leprini A, Zardi L. Transforming growth factor-beta regulates the splicing pattern of fibronectin messenger RNA precursor. FEBS Lett. 1990; 261:175–178. [PubMed: 2307231]

29. Togo S, Shimokawa T, Fukuchi Y, Ra C. Alternative splicing of myeloid IgA Fc receptor (Fc alpha R, CD89) transcripts in inflammatory responses. FEBS Lett. 2003; 535:205–209. [PubMed: 12560105]

30. Cowling RT, et al. Effects of cytokine treatment on angiotensin II type 1A receptor transcription and splicing in rat cardiac fibroblasts. Am J Physiol Heart Circ Physiol. 2005; 289:H1176–1183. [PubMed: 15879490]

31. Creusot RJ, et al. Tissue-targeted therapy of autoimmune diabetes using dendritic cells transduced to express IL-4 in NOD mice. Clin Immunol. 2008; 127:176–187. [PubMed: 18337172]
Fig. 1. PTA and Deaf1 isoform expression in the PLN of NOD mice. a) Expression of pancreatic PTAs in the PLN of NOD versus NOD.B10 mice, as assessed by microarray analysis. b) Deaf1 mRNA expression in the PLN of NOD mice, as detected by Probe 1 and Probe 2 using microarray analysis. Data in panels a and b represent the mean log ratio of (NOD/NOD.B10) ± s.d. (n = 10). c) Two isoforms of Deaf1 (DF1 and DF1-VAR1) were cloned from the PLN of 12 week old NOD mice. DF1 hybridizes to Probe 1 and 2, while DF1-VAR1 only hybridizes to Probe 2. DF1-VAR contains a deletion in the N-terminal alanine-rich region and a partial intronic insertion between exon 6 and 7 that introduces a premature stop codon. Abbreviations: Ala (alanine); SAND (Sp100,AIRE-1-NucP41/75,DEAF-1); NLS (nuclear localization signal); NES (nuclear export signal); Zf-MYND (zinc finger-Myeloid, Nervy, and DEAF-1).
Fig. 2.
Quantification of DF1 and DF1-VAR1 gene expression in NOD PLN. DF1 (a), DF1-VAR1 (b) and Gapdh (c) gene expression was measured in 4, 12, and 20 week NOD and NOD.B10 PLN samples. (d) DF1, DF1-VAR1 and Gapdh mRNA was measured in 12 week NOD and NOD.B10 spleen samples. Gene expression was normalized to Actb mRNA. Values represent the mean ± SEM. *P < 0.05. P-values were determined using the Student’s unpaired t-test, two-tailed. n ≥5 for all groups.
Fig. 3.
Cellular localization, hetero-dimerization and transcriptional activity of DF1 and DF1-VAR1. 

a) Immunoblot of HEK 293 whole cell lysates transfected with 1 μg of DF1-Flag, DF1-VAR1-EGFP, 1 μg of both plasmids or empty control plasmid. Both DF1 (~70 kDa) and DF1-VAR1 (~50 kDa) were recognized by the Deaf1 antibody used. Cyclophilin B expression was assessed as a loading control.

b) Confocal images showing the subcellular localization of EGFP-tagged DF1 and turbo-RFP-tagged DF1-VAR1 in DAPI-stained HEK 293 cells. Scale bar = 20 nm; images are representative of more than 100 similar cells.

c) Immunoblot of nuclear lysates extracted from HEK 293 cells transfected with 0 or 0.5 μg of Yip et al. Page 13 Nat Immunol. Author manuscript; available in PMC 2010 March 01. Yip et al. Page 13 Nat Immunol. Author manuscript; available in PMC 2010 March 01.
DF1-Flag and/or DF1-VAR1-EGFP. The nuclear marker histone H3 expression was assessed as a loading control. The densiometric ratio of DF1-VAR1 to histone H3 for each of these samples is shown below. Data is representative of 4 separate experiments. d) Co-immunoprecipitation assay. HEK 293 cells were transfected with the indicated Flag- or V5-tagged DF1 constructs. Lysates were subjected to immunoprecipitation and immunoblot with the indicated antibodies. e,f) Transcriptional activity of DF1 and DF1-VAR1 (or empty EGFP control vector) alone or in combination, as assessed using the 26 bp Deaf1-response element-luciferase reporter plasmid. Data in panel e-f are representative of similar results obtained in 3 separate experiments.
Fig. 4.

Analysis of Deaf1-KO mice. (a) Microarray analysis was used to identify genes that are upregulated and downregulated in the PLN of Deaf1-KO mice. Graph shows fold change in gene expression in PLN of BALB/c Deaf1-KO compared to BALB/c wild-type mice. \( n = 2 \) (b,c). Quantitative PCR (QPCR) was used to measure gene expression differences in BALB/c Deaf1-KO (filled bars) versus BALB/c wild-type PLN (white bars; b) and thymus (c). Gene expression was measured in triplicate by QPCR and normalized with 18S rRNA expression. Values represent the mean ± SEM, \( n = 4 \) in each group. * \( P \leq 0.001 \); ** \( P \leq 0.01 \), Student’s unpaired t-test, two-tailed. d) Serum of 30 wk old Deaf1-KO or wild-type BALB/c mice was used for immunoblotting of mouse eye lysates. Data are representative of 3 similar experiments. e) Confocal images of indirect immunofluorescence experiments performed in the mouse eye. Tissue sections (5 μm) were incubated with diluted serum (1:100) from Deaf1-KO or wild-type littermate controls or with no serum (negative control), followed by Alexa-488 conjugated anti-mouse IgG (1:2000). A high magnification image of the boxed region is shown in the top right panel. (Abbreviations: OS = outer segment; OP = outer plexiform; C = choroid; ON = outer nuclear layer; IN = inner nuclear layer). Data is representative of 12 sections per group.
Fig. 5.
Deaf1 regulates gene expression in NIH 3T3 cells. NIH 3T3 cells were co-transfected with Deaf1 or nonsense siRNA and with DF1-Flag. (a) The expression of exogenous Deaf1 was assessed by immunoblot with anti-Flag. A non-specific band (N.S.) was used as a loading control. (b, c) Expression of the indicated endogenous mRNA transcripts was measured by QPCR at indicated times after transfection (b) or at 48 h after transfection (c). Cells transfected with nonsense siRNA and Deaf1 siRNA are indicated by white and grey bars, respectively. Gene expression was measured in triplicate by QPCR and normalized with 18S rRNA expression. Values represent the mean ± SEM of 3 separate experiments. *P ≤ 0.001; **P ≤0.05, Student’s unpaired t-test, two-tailed.
Regulation of PTA expression in NOD PLN and LN CD45^− cells by Deaf1 and DF1-VAR1.

a) Gene expression of PTAs in the PLN of NOD versus NOD.B10 mice at various ages (n ≥ 4 per group). Data are normalized with 18S rRNA expression and presented as a percent of age-matched NOD.B10 controls.

b) Relative expression of DF1-VAR1 mRNA in various cells isolated from the 12-week NOD PLN (n = 3). Expression was normalized to Actb.

c) DF1 mRNA expression in LN CD45^− cells transfected with Deaf1 or nonsense siRNA (n = 3) was measured by QPCR at the indicated times after transfection.

d) LN CD45^− cells were transfected with Deaf1 or nonsense siRNA and expression of the indicated mRNA...
transcripts was measured by QPCR 48 h after transfection (n ≥4). For c and d, cells transfected with nonsense siRNA and Deaf1 siRNA are indicated by white and grey bars, respectively. e) LN CD45− cells were infected with viral particles carrying DF1-VARI (grey bars) or an empty control plasmid (white bars), and mRNA expression was measured 72 h later (n = 3). Gene expression was measured in triplicate and normalized with 18S rRNA expression. Values represent the mean ± SEM. ***P ≤0.001; **P ≤0.01; *P ≤0.05, Student’s unpaired t-test, two-tailed. f) HEK 293 cells were transfected with DF1-Flag and/or DF1-VARI-EGFP. Cytoplasmic and nuclear extracts were prepared and analyzed by immunoblot. The cytoplasmic marker Gapdh and nuclear marker histone H3 were used as loading controls. Data are representative of 4 similar experiments.
Fig. 7.
Identification and characterization of an alternatively spliced DEAF1 isoform in human PLN. 

a) Two DEAF1 isoforms were cloned from human PLN, a canonical DEAF1 transcript (Hu-DF1) and an alternatively spliced variant (Hu-DF1-VAR). 

b) Confocal images showing the subcellular localization of EGFP-tagged Hu-DF1 and turbo-RFP-tagged Hu-DF1-VAR in DAPI-stained HEK 293 cells. Scale bar = 30 nm; images are representative of more than 100 similar cells.  
c) The transcriptional activity of Hu-DF1 and Hu-DF1-VAR was assessed in HEK 293 cells using the 26 bp Deaf1-response element-luciferase reporter plasmid. Data
are representative of 2 similar experiments. (d,e) HEK 293 cells were transfected with *Hu-DF1-turboRFP* and/or *Hu-DF1-VAR-EGFP*. Cytoplasmic and nuclear extracts were prepared and analyzed by immunoblot. The cytoplasmic marker GAPDH and nuclear marker histone H3 were used as loading controls. Data are representative of 3 similar experiments. f) Quantification of *Hu-DF1-VAR* gene expression in the PLN of healthy and T1D patients by QPCR. \(n = 5; *P < 0.02\), Mann Whitney test, two-tailed.)
Table 1

INS gene expression in the PLN and SPL of T1D and control patients

| Group   | Age | Sex | PLN* | SPL |
|---------|-----|-----|------|-----|
| Control | 24  | F   | +    | n/a |
| Control | 30  | M   | +    | n/a |
| Control | 32  | F   | −    | n/a |
| Control | 30  | M   | +    | +   |
| Control | 41  | M   | +    | +   |
| T1D     | 32  | M   | −    | +   |
| T1D     | 76  | M   | −    | +   |
| T1D     | 37  | F   | −    | +   |
| T1D     | 28  | F   | −    | +   |
| Pre-T1D | 7   | M   | −    | +   |

* QPCR threshold cycles of > 37 were considered negative. Measurements were performed in triplicate and data are representative of ≥ 2 separate experiments.