MONOGENIC MODEL FOR AUTOIMMUNE DISEASES: MOLECULAR BASIS OF AUTOIMMUNE POLYENDOCRINOPATHY - CANDIDIASIS - ECTODERMAL DYSTROPHY (APECED)

Maria Halonen

Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland and
Department of Medical Genetics, University of Helsinki, Helsinki, Finland, and
Hospital for Children and Adolescents, Helsinki University Hospital, Helsinki, Finland

Academic dissertation

To be publicly discussed by the permission of the Medical Faculty of the University of Helsinki,
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Supervised by:

Docent Ismo Ulmanen
National Public Health Institute
Helsinki, Finland

and

Professor Leena Peltonen
University of Helsinki
National Public Health Institute
Helsinki, Finland

Reviewed by:

Docent Pärt Peterson
Institute of Medical Technology
University of Tampere
Tampere, Finland

and

Professor Irma Thesleff
Institute of Biotechnology
The Academy of Finland
University of Helsinki
Helsinki, Finland

To be publicly discussed with:

Professor Raimo Voutilainen
Department of Pediatrics
University of Kuopio
Kuopio, Finland

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Kansanterveyslaitos (KTL)
Mannerheimintie 166
00300 Helsinki
p. vaihde 09-47441, telefax 09-4744 8408

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsinki
tel. växel 09-47441, telefax 09-4744 8408

National Public Health Institute
Mannerheimintie 166
00300 Helsinki, Finland
phone +358-9-47441, telefax +359-9-4744 8408

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”If we were not able or did not desire to look in any new direction, if we did not have a doubt or recognize ignorance, we would not get any new ideas. There would be nothing worth checking, because we would know what is true. So what we call scientific knowledge today is a body of statements of varying degrees of certainty. Some of them are most unsure; some of them are nearly sure; but none is absolutely certain. Scientists are used to this. We know that it is consistent to be able to live and not know. Some people say, ”How can you live without knowing?” I do not know what they mean. I always live without knowing. That is easy. How you get to know is what I want to know.”

Richard Feynman, The meaning of it all

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

1. M. Halonen, P. Eskelin, A-G. Myhre, J. Perheentupa, E.S. Husebye, O. Kämpe, F. Rorsman, L. Peltonen, I. Ulmanen and J. Partanen, AIRE mutations and Human Leukocyte Antigen Genotypes as Determinants of the Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy Phenotype, J. Clin. Endocrinol. Metab. 87(6), 2568-2574, 2002

2. M. Halonen, M. Pelto-Huikko, P. Bjorses, L. Peltonen, I. Ulmanen, M. Kolmer; Subcellular location and expression pattern of autoimmune regulator (Aire)- the mouse ortholog for human gene defective in Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy (APECED), J. Histochem. Cytochem., 49: 197-208, 2001

3. P. Bjorses, M. Halonen, J. Aaltonen, J. Palvimo, I. Ulmanen, Leena Peltonen; Mutations in the AIRE gene: effects on subcellular localization and transactivation function of APECED protein, Am.J.Hum.Gen. 66:378-392, 2000

4. M. Halonen*, H. Kangas*, J. Ollila, T. Meriluoto, M. Kolmer, M. Vihinen, J. Palvimo, J. Saarela, I. Ulmanen and P. Eskelin; Functional characterization of the AIRE protein, defective in patients with APECED, submitted

*These authors contributed equally to this work

ABBREVIATIONS

aa	amino acid
AADC	aromatic L-amino acid decarboxylase
AID	autoimmune disease
AIR	adaptive immune response
AIRE	the autoimmune regulator protein
AIRE	the autoimmune regulator gene
ALPS	autoimmune lymphoproliferative syndrome
APC	antigen-presenting cells
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS	autoimmune polyglandular disease
CBP	CREB-binding protein
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
DBD	DNA-binding domain
DC	dendritic cells
DEAF-1	deformed epidermal autoregulatory factor-1 of Drosophila melanogaster
DISC	death-inducing signalling complex
DP	double positive (thymocytes)
GAD	glutamic acid decarboxylase
GST	Glutathione S-transferase
H+K+ATPase	the proton pump of the gastric mucosa
HAS	heat stable antigen
HEL	hen egg lysozyme
HLA	human leukocyte antigen
HSR	homogeneously staining region
ICA	slet cell antigen
ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IIR	innate immune response
IL	interleukin
IPEX	immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
kb	kilobase pair
kD	kilodalton
LFA	leukocyte functional antigens
mAire	the mouse Aire protein
mAire	the mouse Aire gene
MHC	major histocompatibility region
mRNA	messenger ribonucleic acid
mTEC	medullary thymic epithelial cells
NLS	nuclear localisation signal
NOD	non-obese diabetic
NUDR	nuclear DEAF-1 related protein
OMIM	online Mendelian inheritance in man
PAMP	pathogen-associated molecular pattern

PD-1	programmed cell death 1
PHD	plant homeodomain
PML	promyelotic leukemia
PRR	pattern recognition receptors
RT-PCR	reverse transcriptase assisted polymerase chain reaction
SAGE	Serial Analysis of Gene Expression
SAND	Sp100, AIRE, NucP41/75 and DEAF-1
Scc	side chain cleaving enzyme
SP	single positive (thymocytes)
TCR	T cell receptor
TG	thyroglobulin
TH	tyrosine hydroxylase
Th cell	T helper cell
Tk	thymidine kinase
TN	triple negative (thymocytes)
TNF	tumor necrosis factor
TPH	tryptophan hydroxylase
TPO	thyroid peroxidase
Treg	T regulatory cells
VP-16-AD	herpes simplex virus VP16 activation domain

SUMMARY

APECED (for Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal dystrophy) provides a unique model for the molecular studies of autoimmune diseases due to the monogenic inheritance of the disease. In several monogenic diseases, the phenotype may be modified by genes other than the disease gene and most autoimmune diseases have a complex genetic etiology. The clinical phenotype of the APECED patients varies between the siblings carrying the same mutations in the *AIRE* (for Autoimmune regulator) gene. Thus, genetic complexity may lie behind the APECED phenotype, but so far the association of the *AIRE* genotype to the APECED phenotype has not been studied, and the genetic factors other than the *AIRE* gene that may modify the phenotype of APECED, have not been identified.

In order to analyse the mutations found in patients with APECED, we collected a series of 150 patients with APECED from various ethnic backgrounds. Among these, eighteen different mutations, small insertions, deletions or one base pair substitutions were identified and each mutation was predicted to lead either to a truncated form of the AIRE protein or to an amino acid change. Nine of the mutations were novel. The mutations were spread throughout the coding region of *AIRE*, yet four evident mutational hot spots were observed, in exon 2 encoding for the HSR (for homogeneously staining region) domain, in exon 6 encoding for the SAND domain, in exon 8 encoding for the first PHD finger and in exon 10 between the coding regions of the first and second PHD zinc fingers. The major Finnish mutation, R257X, representing the founder mutation in the Finnish population, was found in 89% of the Finnish disease alleles. This mutation was also present in 33% of the non-Finnish disease alleles on several haplotype backgrounds suggesting independent origins for the mutation. Interestingly, a common founder mutation, Y85C in the HSR domain of AIRE, was found for the Iranian Jewish APECED patients.

Furthermore, to analyse the genetic factors determining the APECED phenotype, we studied the *AIRE* and human leucocyte antigen (HLA) class II genotypes in a series of 104 index patients with APECED. The presence of some association between the type of mutation in the *AIRE* gene and the APECED phenotype was shown: the frequency of mucocutaneous candidiasis was lower in the patients without the R257X allele. In addition, the phenotype of APECED was shown to be modified by other genetic elements besides the *AIRE* gene alleles. The HLA class II alleles, DRB1*03 and 04 seemed to predispose to, and the DRB1*15 to protect from particular phenotype components. These same allelic associations have previously been found in autoimmune alopecia, isolated Addison's disease, Addison's disease as part of Autoimmune polyglandular disease type 2 (APS 2), and isolated type 1 diabetes, suggesting similarities in the pathogeneses. In future, the characterisation of other modifying factors will be clinically important in understanding the clinical features and their progress in patients with APECED.

In order to reveal the pathogenesis of APECED and the putative role of AIRE-associated

pathways in more common autoimmune diseases, an understanding of the functions of the AIRE protein is essential. First, in order to analyse the biological similarities of the human *AIRE* and mouse *Aire* genes (from here on the mouse *Aire* is called *mAire*), the *mAire* cDNA was cloned and expressed in cultured cell lines. The mAire protein was localised in nuclear dots, cytoplasmic filaments and aggregates, closely resembling the subcellular localisation pattern of the human counterpart. Thus, it seems that the *mAire* represents an orthologue of the *AIRE* gene with similar biological role(s) and provides a good model for the molecular studies of APECED. Next, in order to provide a basis for the functional studies of the mAire protein, the tissue expression pattern of the *mAire* gene was studied using several methods. The *mAire* gene expression was seen in multiple immunologically relevant tissues such as the thymus, spleen, lymph node, and bone marrow as well as in various non-immunological tissues such as the kidney, testis, adrenal glands, liver, and ovary. The findings suggest that the mAire protein controls autoimmunity either by regulating the central and/or the peripheral mechanisms of tolerance. Furthermore, mAire may have function(s) outside the immune system.

The AIRE protein is characterised by several domains found in transcriptional regulators. To explore the transactivation potential of the AIRE protein, a reporter gene assay was used. AIRE activated the transcription of the reporter gene by strongly stimulating the promoters tested. For the first time, this provided evidence for the transcriptional transactivator function of AIRE. Two truncated mutant proteins encoded by the cDNAs carrying APECED-causing mutations showed no activity in the assay, whereas protein with a disrupted first plant homeodomain type (PHD) finger but intact second PHD finger showed about one third of the wild type activity. To further analyse the functional roles of the domains of the AIRE protein, the subcellular localization, transactivation capacity, homomultimerisation and complex formation of the isolated domains of the mAire polypeptide and several AIRE polypeptides carrying APECED mutations, were studied. Most patient mutations altered the nucleus-cytoplasm distribution of AIRE and affected its association with nuclear dots and cytoplasmic filaments. The zinc fingers were responsible for the transactivation capacity of AIRE. Other regions of AIRE modulated this function, and consequently all the patient mutations decreased the transactivation capacity. The HSR domain displayed the homomultimerization activity of AIRE and all the APECED-causing missense mutations of the HSR and SAND domains, but not mutations in the other domains, decreased this activity. In conclusion, the results suggest that the amino acids on the predicted surface of the HSR domain mediate the nuclear export of AIRE. Additionally, the association of AIRE with nuclear dots correlates with the transactivation capacity of AIRE. Interestingly, in cellular lysates the AIRE protein was present in soluble high molecular weight complexes, and mutations in the HSR and PHD domains disturbed the formation of these complexes.

INTRODUCTION

The function of the immune system is to recognise and kill pathogenic micro-organisms entering the body. The human immune system is extremely efficient and complex. One of the most critical tasks of the immune system is to avoid attacking self molecules. The capacity of the immune system to be specifically unresponsive to an antigen is referred to as the immunological tolerance. The breakdown of the immunological tolerance leads to autoimmune disease (AID), in which a sustained autoimmune attack is directed to a specific antigen(s) that is/are either confined to a particular organ or to an antigen(s) that is/are widely present in the body. Many fundamental issues in the molecular etiology of autoimmune diseases are still to be answered.

The AIRE protein provides an interesting and unique model for the molecular studies of autoimmunity, as the defective form of the protein is the cause of a rare recessive monogenic autoimmune disease APECED. Only few other monogenic autoimmune diseases, such as autoimmune lymphoproliferative syndrome (ALPS) types I and II and X-linked immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), are known.

APECED is caused by mutations in the autoimmune regulator (*AIRE*) gene on chromosome 21q22.3. The most common components of the disease are chronic mucocutaneous candidiasis, hypoparathyroidism, and Addison's disease, but several other endocrine deficiencies and ectodermal dystrophies also occur, and the phenotype varies widely.

The purpose of this study was to understand the genetic factors determining the phenotype of APECED. Another goal was to provide a molecular basis for the further studies of the pathogenesis of APECED by analysing the tissue expression pattern of the mouse *Aire* gene and by characterising the domains of the AIRE protein by utilising mutational analysis of AIRE and the individual domains of the mouse Aire protein.

REVIEW OF THE LITERATURE

1.Immune system and autoimmunity

The human immune system is very complex and includes various effector cells. The first line of defense against microbes are the mechanical barriers to the microbes, and the human barriers include the skin and the bacterial normal flora, the epithelial cilia of the respiratory tract, tears, saliva, urine, gastric and bile acids and the pancreatic enzymes. Traditionally, the immune response has been divided into two major categories: the innate and adaptive immune response (IIR and AIR, respectively). The two immune response mechanisms are now known to be closely interrelated (Ochsenbein and Zinkernagel 2000). The AIR and IIR have several characteristic features. The molecules of the IIR or their similar forms are ancient (Hoffmann et al. 1999), whereas those of the AIR have evolved more recently (Matsunaga and Rahman 1998). The genes encoding for the effector molecules (T cell receptor (TCR) and immunoglobulin (Ig)) of the AIR are rearranged during the maturation of these molecules, whereas the genes expressing the effector molecules for the IIR are encoded in the germline DNA and do not require gene rearrangements (Janeway and Medzhitov 2002). The cells of the AIR that belong to the same class, are distinct from each other (clonal), whereas those of the IIR are identical within one class (non-clonal). The IIR functions with a wide variety of cell types such as the natural killer cells, granulocytes, macrophages and dendritic cells and the complement system. The AIR functions with cells such as the dendritic cells, naïve T cells, cytotoxic T cells (CD8+), helper T cells (CD4+ Th1/Th2), natural killer like T cells, regulatory T cells (CD4+ CD25+) and B cells. The major role of the IIR is to kill the bulk of pathogenic bacteria and viruses entering the body. Those that are not killed, are specifically recognised and destroyed by the AIR. The price which is paid for the effectiveness of the AIR includes allergy, autoimmunity and rejection of tissue grafts (Janeway and Medzhitov 2002). Autoimmunity arises when the immune system attacks self molecules and to prevent this, the immune system makes a distinction between self and nonself (Bretscher and Cohn 1970; Lafferty and Cunningham 1975; Jenkins and Schwartz 1987; Janeway et al. 1989; Janeway 1992; Janeway 2002; Medzhitov and Janeway 2002). According to another model, instead of recognition between self and nonself, the immune system distinguishes between entities that are harmless and those that do damage (Matzinger 1998; Matzinger 2001a; Matzinger 2001b; Matzinger 2002).

1.1 Activation of the immune system

The activation of the immune response of the AIR requires signalling from effective antigen presenting cells. These can be formed from macrophages, B cells or dendritic cells. Based on the relevance for AIRE, which has been shown to be expressed in both peripheral and thymic dendritic cells (Heino et al. 2000; Kogawa et al. 2002b), the following will concentrate on dendritic cells (DCs). The DCs represent antigen-presenting cells (APC) with a capacity to induce a primary immune response by activating naïve T cells, but probably also B and natural killer cells (Hart 1997; Banchereau and Steinman 1998; Banchereau et al. 2000; Hartgers et al.

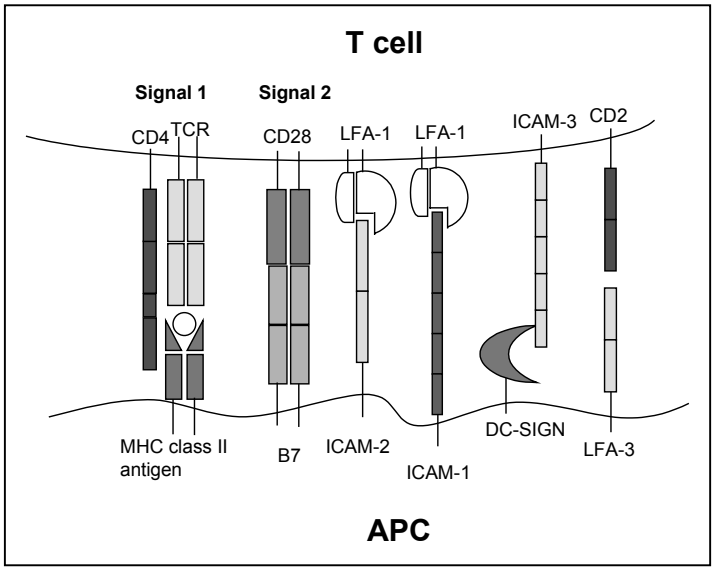
2000; Guermonprez et al. 2002; Lipscomb and Masten 2002). The DCs, first characterised in the 1970s (Steinman et al. 1975), represent a heterogeneous group of cells with a common precursor (Spits et al. 2000; Shortman and Wu 2001; Cavanagh and Von Andrian 2002). The maturation of the DCs into antigen-presenting cells is triggered by two types of signals from pathogens and/or surrounding tissue; (i) by exogenous signals from pathogen-associated molecular patterns (PAMPs), which are products of microbial physiology with a conserved molecular structure and which bind to specific pattern recognition receptors (PRRs) on the DCs (Medzhitov and Janeway 2002), or, (ii) by different endogenous danger signals. In order to become efficient T cell stimulators and to activate the adaptive immune system, the DCs begin to mature after stimulation by activating signals. Upon maturation, the expression of endocytic/phagocytic receptors of the DCs is abolished and the costimulatory molecules such as CD40 (CD for cluster of differentiation), CD58, CD80 and CD86 are upregulated. Further, the morphology of the DCs changes: the adhesive structures are lost, the cytoskeleton is reorganised and the cell motility increases. The maturing DCs leave the inflamed tissue and enter the T cell zones in the paracortical area of the lymph node through the lymphatic vessels (Hart 1997; Banchereau and Steinman 1998; Banchereau et al. 2000; Hartgers et al. 2000; Guermonprez et al. 2002; Lipscomb and Masten 2002).

The mature dendritic cells, now effective APCs, can induce the activation of the cells of the AIR such as naïve T cells, of the IIR such as the natural killer cells and the natural killer like T cells (Godfrey et al. 2000), that cannot be clearly classified as cells of the AIR or of the IIR. The following will concentrate on the activation of the T cells. The naïve T cells circulate continuously from blood to lymphoid organs and back, and make contact with thousands of antigen presenting cells every day (the development of naïve T cells from a precursor is described in section 1.2.1). The high number of contacts is essential, as only one naïve T cell in 10^4-10^6 is likely to be specific for a particular antigen. In addition, these contacts provide survival signals for the naïve T cells (Freitas and Rocha 2000). In order to be activated and develop into effector T cells, naïve T cells must receive two signals from antigen presenting cells, a process called T cell priming (Figure 1) (Albert et al. 2001; Shortman and Heath 2001).

The proliferation of the naïve T cell is promoted by the synthesis of interleukin-2 (IL-2) by the T cell, the expression of which is triggered by the costimulatory signal 2 (Figure 1). The uncommitted naïve CD4+ T cell will first proliferate into an immature effector T cell (Th0, Th for T helper cell). Various types of signals determine the polarisation of the Th0 cell to either Th1 or Th2 type cell: (i) the signals from dendritic cells, particularly the production of different types of cytokines by different subpopulations of dendritic cells, (ii) the tissue-specific environmental factors, (iii) the ratio of APCs to T cells and (iv) the duration of the interaction between a T cell and an APC (Guermonprez et al. 2002). In contrast, the CD8+ T cell is predestined to become a cytotoxic T cell upon activation. The mature T cells, called effector T cells, have distinct functions. The Th1 cells are involved in the cell-mediated immunity, and they function by activating macrophages and inducing B cells to produce opsonising antibodies by producing IFN-γ (IFN for interferon), TNF-β (TNF for tumor necrosis factor), TNF-α, CD40 ligand, Fas

ligand, and IL-3. The Th2 cells are involved in the humoral immunity and they activate B cells to make neutralising antibodies by producing e.g. IL-4, IL-5 and CD40 ligand. In contrast, the CD8 cells kill their target cells by direct cell-contact with the help of perforin, granzymes or Fas ligand (Janeway et al. 2001a).

Figure 1. DC – T cell interaction. Signal 1 involves the recognition of the TCR and its specific antigen presented on the MHC of the mature APC. Signal 2 involves the interaction between costimulatory molecules (in particular the glycoproteins CD80 (= B7.1) and CD86 (=B7.2)) on the dendritic cell and, their ligands on the naïve T cell (CD28) (Hart 1997; Banchereau and Steinman 1998; Banchereau et al. 2000; Hartgers et al. 2000; Guermonprez et al. 2002; Lipscomb and Masten 2002). In addition, the interaction of the T cell and APC is mediated by several other molecules on the APC (ICAM-1 (ICAM for intracellular adhesion molecules), ICAM-2, DC-SIGN, LFA-3 (LFA for leukocyte functional antigens) and on the T cell (LFA-1, ICAM-3, CD2). Many other molecules on the APC, such as tumor necrosis factor ligands and receptors (CD40, OX40L, 4-IBBL, TRANCE (RANK), CD27, CD30L) also provide costimulatory signals to the T cells (via CD40L, OX40, 4-1BB, TRANCE-L, CD27L, and CD30, respectively).



Modified from (Janeway et al. 2001a)

1.2 Regulation of immunological tolerance and its breakdown

Autoimmune diseases are caused by the breakdown of immunological tolerance, which refers to specific unresponsiveness to an antigen (Bellgrau and Eisenbarth 1999; Mackay 2000; Lesage and Goodnow 2001; Leng and Bentwich 2002; Ohashi 2002; Ohashi and DeFranco 2002). Upon contact with APC, the lymphocyte decides between tolerance and immunity/autoimmunity. Immature APCs induce tolerance in the absence of a costimulus, but in case of a sudden release of self antigens and maturation signals for APC, the APCs may present self-antigens to autoreactive naïve T cells, which are consequently activated. All individuals carry autoreactive lymphocytes, but only a small fraction of us develop autoimmune diseases, which result from a sustained autoimmune attack against target organs. The development of autoimmune diseases is mediated by the composition of the T and B cell

repertoire by central mechanisms of tolerance and by the control of autoreactive lymphocytes by peripheral mechanisms of tolerance. The control of the CD4+ T cells is specifically important, as these can activate the rest of the immune system. The following describes the regulation mechanisms of the T cells.

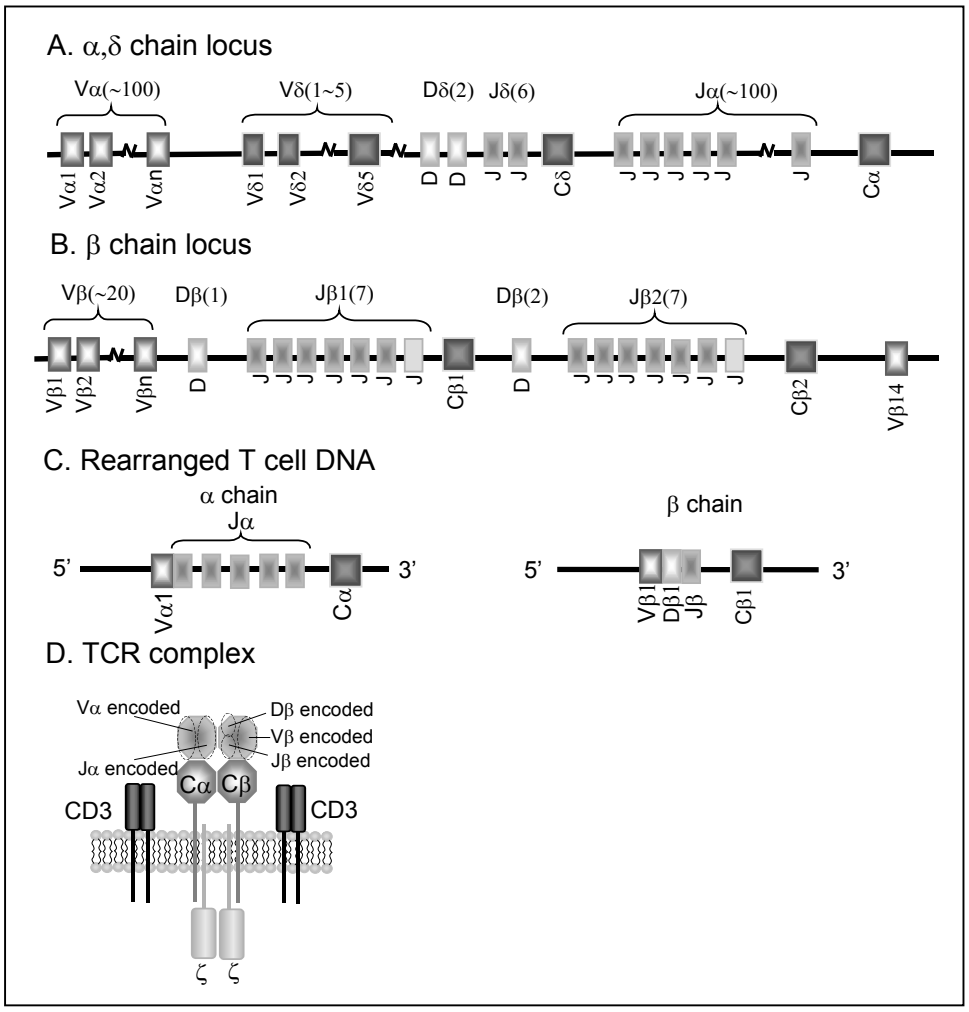
1.2.1 Mechanisms of central tolerance

The central tolerance is induced by negative selection of cells that can recognise self structures (i.e. autoreactive) in lymphopoietic organs. The T cell development takes place in the thymus, where the T cell repertoire of an individual is selected (Figure 2 and 3). The aims of the T cell development are to (i) commit to become a T cell, (ii) rearrange the T cell receptor (TCR) encoding genes to generate a functional TCR, (iii) decide upon becoming either an α:β or γ:δ cell, (iv) choose to express either CD8 or CD4, (v) select the TCR positively, so that only TCRs that bind to self-antigen presenting MHC molecules with low affinity and avidity, are maintained and (vi) select TCR negatively, so that autoreactive T cell clones are deleted (Paul 1998d). The thymic tissue consists not only of developing T cells and a heterogeneous population of stromal cells, but also of connective and nervous tissue, blood and lymphatic vessels and even of primitive muscle cells. The stromal cells of the thymus include epithelial cells (from the ecto- and endoderm) and mesenchymal cells (from the mesoderm) such as fibroblasts, macrophages, dendritic cells and some B cells. The thymus consists of three functionally and architecturally distinct layers: the subcapsular region, the cortex and medulla (Paul 1998d; Res and Spits 1999). Crosstalk between the cells of the stroma and developing thymocytes is necessary for the proper development of the thymic layers (Ritter and Boyd 1993; van Ewijk et al. 1994; van Ewijk et al. 1999). The size of the thymus diminishes considerably during puberty, and much of the tissue is replaced by fibrous tissue and fat. However, the differentiation of the T cells continues even after puberty (Jamieson et al. 1999). Only a small percentage of the undifferentiated T cell progenitors that enter the thymus ever exit to the periphery as naïve T cells.

The deletion of autoreactive thymocytes by negative selection is thought to take place within the medulla and/or corticomedullary junction of the thymus (Hoffmann et al. 1992; Laufer et al. 1999; Naquet et al. 1999), however, this assertion has been a subject of debate. The medulla is inhabited by SP (for single positive) thymocytes that are at different stages of maturation. These thymocytes can be monitored with lymphocyte markers such as the heat stable antigen (HSA) molecule, which is expressed in the immature or semimature forms of SP lymphocytes, but is absent in the fully mature thymocytes (Kishimoto and Sprent 2000). The semimature SP thymocytes with very high affinity to self antigens are deleted by negative selection when they come into contact with activated APCs, which can be (i) hematopoietic-derived cells or (ii) subpopulations of medullary epithelial cells (mTEC). The costimulation provided by CD80/86 on the APC and received by CD28 on the thymocyte, induces apoptosis, whereas in the periphery, similar ligation promotes the proliferation of a T cell into an effector cell. However, if the SP thymocytes are no longer expressing the HSA molecule, the ligation leads to a proliferation resembling that seen in the periphery. The apoptosis induced by negative selection is mediated

by death receptors other than Fas, but the Fas pathway is important in the deletion of a subpopulation of semimature SP cells that are ligated with a large amount of antigen (Sebzda et al. 1999; Kishimoto and Sprent 2000).

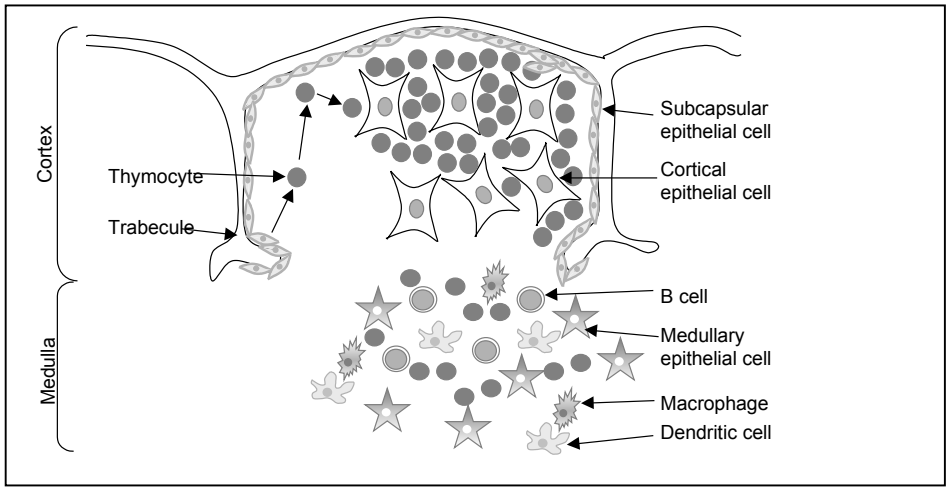
Figure 2. The T cell antigen receptor (TCR). TCR determines the specificity of each T cell, and although most T cells carry one unique TCR, some cells may express two different TCRs (Padovan et al. 1993). **A.** The loci on chromosome 14q11.2 encoding for the variable (V), diversity (D), joining (J) and constant (C) parts of the α and δ chains of the TCR molecule are overlapping. The number in brackets indicates the number of encoding genes. **B.** The locus on chromosome 7q34 encodes for the β chains of the TCR. The last of the set of seven J genes are noncoding pseudogenes. **C.** The recombination of the β locus occurs prior to that of the α locus. The recombined DNA encodes for the α and β chains of the TCR. **D.** The TCR is composed of two highly variable chains, α and β (95%), or γ and δ, which are members of the immunoglobulin protein superfamily. The α:β chains of the TCR are associated with the intermembrane CD3 molecule, which accounts for the intracellular signalling cascades of the TCR. In addition to CD3, the α:β chains are associated with a ζ homodimer.



Modified from (Roitt et al. 1998; Janeway et al. 2001a)

Figure 3. A. The structure and the most common cell types found in the thymus. **B.** The differentiation of thymocytes. Generally, four subpopulations of developing thymocytes representing the different stages of thymocyte maturation are found in the thymus and, these can be identified by their profiles of surface marker expression. The different maturation stages take place in distinct microenvironments. The first subpopulation, the undifferentiated T cell progenitors can give rise to NK cells, B cells and dendritic cells. The progenitor cells develop into the second subpopulation, the CD4- CD8- (double negative, DN) cells, which are further subdivided into (i) triple-negative (TN) cells that express no TCR, (ii) γ:δ TCR expressing cells, (iii) α:β expressing cells. The TN cells constitute the major group of DN cells and is made up of CD44+25-, CD44+25+, CD44lo25+ and CD44-25- subpopulations, characterized e.g. by their TCR-β status or specifically, by the expression of the β-chain on the cell surface. The next stage of the thymocyte development and the third subpopulation of thymocytes consists of cells at the double positive (DP) stage. In fact, the majority of the developing thymocytes (80%) express both CD4 and CD8 on their cell surfaces. During this stage, the TCR-α chain of the cells are rearranged and most cells express the TCR complex containing CD3 and α:βTCR. In the last stage of the thymocyte development, the fourth subpopulation of thymocytes is formed by cells that express only CD4 or CD8 and these are called single positive (SP) cells. Some of the SP cells express only low levels of CD3 and no detectable TCR, whereas other cells express high levels of TCR and CD3 complex. The latter cells coexpress several surface markers including heat stable antigen (HSA), the receptor for peanut agglutinin (PNAr), and the activation molecule CD69. A small proportion of the cells in the SP pool represent those cells that have returned from the periphery back to the thymus (Paul 1998d; Kruisbeek 1999; Laufer et al. 1999; Sebzda et al. 1999).

A.



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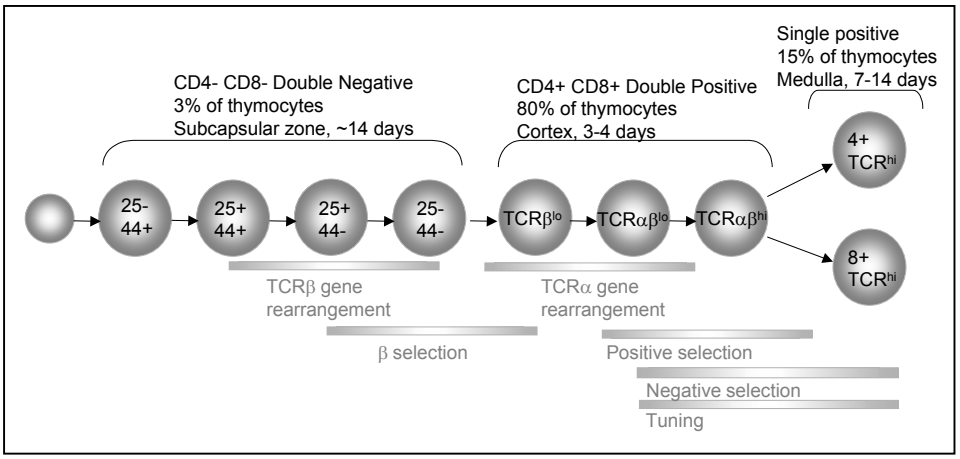


Modified from (Paul 1998d; Sebzda et al. 1999)

A particularly interesting feature of the mTECs, which function in the negative selection of autoreactive thymocytes, is their promiscuous expression of rare tissue-specific antigens (Table 1) (Antonia et al. 1995; Smith et al. 1997; Hanahan 1998; Throsby et al. 1998; Klein and Kyewski 2000; Derbinski et al. 2001; Pugliese et al. 2001; Kyewski et al. 2002). In particular, the mTECs, but possibly also APCs of hematopoietic origin, represent the specialised cell type for ectopic expression of antigens, although this matter is still under investigation. The ectopic expression of antigens in mTECs allows the deletion of not only those thymocytes that are autoreactive against ubiquitously expressed proteins and against abundant blood-borne self-antigens, but also of those that are autoreactive against various ectopically expressed proteins (Hanahan 1998). However, the ectopic expression in the thymus seems to be sufficient, but not necessary for the induction of tolerance (Derbinski et al. 2001). This, in turn, suggests that ectopic expression in mTECs has evolved to anticipate the existence of peripheral regulation mechanisms of tolerance (Derbinski et al. 2001). Furthermore, the mTECs may play an important role in the selection of T regulatory cells (Klein and Kyewski 2000).

Modified from (Kyewski et al. 2002)

Expressed gene	mTECs	Dendritic cells
α1-Antitrypsin	+	-
α1-Crystallin	+	+
α-Fetoprotein	+	-
Albumin	+	-
Amylase1	+	-
Complement C5	+	+
CRP	+	-
Elastase	+	-/+
Erythropoietin	+	-
GAD56	+	-
GAD67	+	-
Gp100	+	+
H/K ATPaseα	+	+
H/K ATPaseβ	+	-
Haptoglobin	+	-
iFABP	+	-
Insulin	+	-
IRBP	+	-
Lactalbumin	+	-
MAGE-a1,2,3,5,6,8	-	-
MAGE-a4	-	-
MAGE-b1,2,3	+	+
MOG	+	-
nAChRα1	+	-
P1A	+	-/+
PLP	+	-/+
Renin	+	-/+
Retinal S-antigen	+	-
S100β	+	+
SAP	+	-
Somatostatin	+	-/+
Thyroglobulin	+	-
Trypsin2	+	-
Tyrosinase	+	-

Table 1. The promiscous expression of rare tissue-specific antigens in mTECs and dencritic cells of the thymus medulla detected by RT-PCR in C57BL/6 mice Kyewski et al. 2002. Abbreviations: nAChRα1=nicotinic acetylcholine receptor α1; CRP=C-reactive protein, Gp100=Melanosomal protein silver/Pmel17/gp87; H/K ATPase=proton pump of the stomach, iFABP=intestinal fatty acid-binding protein,; IRBP, interphotoreceptor retinoid-binding protein; MAGE=melanoma antigen gene; MOG=myelin oligodendrocyte glcoprotein; PLP=proteolipid protein; SAP=serum amyloid P component. -/+ refers to no or. weak signal in duplicate analysis.

1.2.2 Mechanisms of peripheral tolerance

Following the deletion of most autoreactive T cells in the thymus, there are still some present in the periphery. If the conditions for the negative selection of thymocytes were too stringent, the repertoire of T lymphocytes might be too narrow in comparison to the range of foreign peptides, and the immune system would fail to respond to pathogens. For this reason, the negative selection is not absolute and some autoreactive thymocytes escape the negative selection and enter the periphery. Other reasons for the presence of autoreactive T-lymphocytes in the periphery include the molecular mimicry (Benoist and Mathis 2001). Some pathogenic antigens resemble those of the host. In this case, an immune response is induced normally by the T cell activation in the lymphoid organs. However, the mature effector T cells are subsequently able to react with self antigens that resemble the original host antigen, and thus, they are able to promote autoreactive immune response.

Several peripheral mechanisms for regulation of immunological tolerance exist. These can be further subdivided into (i) T cell intrinsic and (ii) T cell extrinsic according to their mode of action (Walker and Abbas 2002). The intrinsic regulatory mechanisms act directly on the responding T cell, causing the ignorance or anergy of the corresponding T cell, phenotypic skewing towards Th1 or Th2 differentiation, or apoptosis of the T lymphocyte (Wang and Lenardo 1997). In contrast, the extrinsic regulatory mechanisms affect other cells than the responding T cell, such as DCs or regulatory T cells.

The restriction of the T cell activation to the lymphoid organs regulates the T cell activation very efficiently. When antigenic stimuli are provided anywhere outside these organs, ignorance or functional inactivation of lymphocytes, called anergy, is induced. Another powerful control mechanism is the induction of ignorance, anergy or apoptosis, if the TCR ligation occurs in the absence of costimulation (Jenkins et al. 1987). Interestingly, at the same time as the medium or high affinity TCR ligation without costimulation causes the inactivation of autoreactive T cell clones, all T cell clones in the periphery seem to continuously undergo low affinity ligations and to receive necessary survival signals from these interactions (Freitas and Rocha 2000). Further, the costimulation and antigen specificities are not enough to activate a T cell, but the amount of the presented antigen must be sufficient to trigger activation. Thus, also the amount of the presented antigen may regulate tolerance (Kurts et al. 1998). More specific mechanisms of intrinsic control of peripheral tolerance include the PD-1 (programmed cell death 1) molecule, which is expressed at high levels in anergic T lymphocytes and may cause anergy by inhibiting cytokine transcription or by inducing cell cycle arrest (Nishimura and Honjo 2001) and, the expression of the tumour suppressor gene *Pten*, which is shown to be important in the regulation of T cell homeostasis (Suzuki et al. 2001). In addition to the regulation of the naïve T cell activation, activated and effector T cells can also be regulated. First, the immune response can be modified to avoid pathogenic effects by for example regulating the differentiation of the Th0 cell towards the Th2 type response, which has been shown to downregulate autoimmunity (Pakala et al. 1997; Walker and Abbas 2002). Further, access of the activated T cells to their targets can be prevented by altering lymphocyte trafficking (Kearney et al. 1994).

An important extrinsic mechanism for the regulation of tolerance is the decision of the DC to mature and become an effective APC (Ohashi and DeFranco 2002). Another readapted concept (Sakaguchi et al. 1995) is the existence of specific regulatory T cell populations, that can suppress activated autoreactive T cells (Maloy and Powrie 2001; Shevach 2002). A minor population of CD4+ T cells coexpress the interleukin-2 receptor (IL-2R) α-chain (=CD25) and these T regulatory (Treg) cells are thought to be important in the control of autoreactive T cells. However, CD25 is generally expressed on activated T cells, so care must be taken in distinguishing between the Treg subpopulation expressing CD25 and the activated T cells. In addition CD4+, but CD25- Tregs may exist (Stephens and Mason 2000). *In vitro* studies of Tregs suggest that (i) suppression of IL-2 production in the responder population, (ii) induction of apoptosis by the interaction between CTLA-4 and CD80/CD86, (iii) latent TGF-β on the surface of the Treg and (iv) the inhibition of expression of costimulatory molecules on APCs may be the mechanisms that account for the suppression of immune response by Tregs. *In vivo* studies of Tregs have been found to be complicated, and suggest that there may be different subpopulations of Tregs which function either by cell-contact dependent mechanisms or, by the secretion of different suppressor cytokines. In conclusion, there are many open questions regarding the function and even the existence of Tregs (Maloy and Powrie 2001; Shevach 2002).

1.3 Effector lymphocytes in autoimmunity

In autoimmune diseases, the target organs are attacked by either autoantibodies and/or T lymphocytes (Santamaria 2001). Their targets may be single or many different antigens, which is/are restricted to a single/multiple organs (Marrack et al. 2001; Santamaria 2001). During the course of the disease, the possibly epitopes of the target molecule(s) multiply (i.e. determinant spreading) (Sercarz 2000). Autoimmune tissue destruction is a very complex process involving various different immune cell types and killing pathways. One of the most common and studied autoimmune diseases is type 1 diabetes, which seems to result from the effect of autoreactive CD4+ and/or CD8+ T cells on the pancreatic insulin-producing β-cells. In Hashimoto's thyroiditis, the destruction of thyroid follicular cells is mainly carried by autoreactive CD8+ T cells. In multiple sclerosis, both CD8+ and CD4+ autoreactive T cells contribute to the autoimmune attack against myelin basic protein. In many diseases, such as Graves disease, myasthenia gravis and systemic lupus erythmatosus, the autoreactive Th2 cells induce the differentiation of B cells into autoantibody-producing plasma cells which are responsible for producing the autoantibodies that account for the autoimmune attack (Santamaria 2001).

2.Autoimmune diseases

The classical definition of an autoimmune disease (AID) includes four criteria (Witebsky E 1957; Rose and Bona 1993; Marmont 1994): (i) the existence of an autoantibody or cell-mediated immunity, (ii) the identification of the corresponding antigen, (iii) the induction of disease in an experimental animal by immunisation with the antigen and (iv) the transfer of disease to a healthy individual by transfer of T-cells, B-cells or autoantibodies. There is a wide spectrum of diseases that can be defined as AIDs using the criteria above. All AIDs seem to be antigen-specific and they are classified according to the target organs and tissues of the autoimmune attack. In organ-specific autoimmune diseases, the autoimmune attack is directed towards an antigen confined to a particular organ, whereas in systemic AIDs the antigen is widely distributed in the body. The organ-specific autoimmune diseases are further subdivided into two groups: destructive, such as type 1 diabetes and non-destructive, such as myasthenia (Paul 1998c; Paul 1998b). The organ-specific AIDs include diseases of the endocrine organs, paraneoplastic disorders, nerve-muscle junction, myelin in central nervous system, uvea of the eye, melanocytes of skin and hair follicles (Eisenbarth 1999), whereas the systemic AIDs characteristically involve the skin, kidney, joints and muscle. Autoimmunity is clinically important, as there are at least 70 known or suspected autoimmune diseases and altogether 3-5% of the general population is affected by these diseases (Marrack et al. 2001). Many of the diseases are common (Table 2) and in the case of all AIDs, women are more frequently affected than men (3:1 sex ratio)) (Mackay 2000; Marrack et al. 2001).

Table 2. The prevalence and autoantigen targets in common autoimmune diseases (Marrack et al. 2001). λs describes the genetic susceptibility of a disease (λs=sibling risk/population frequency) (Vyse and Todd 1996), (OMIM).

Disease	λs	Organ	Autoantigens	Prevalence (%)
Autoimmune gastritis		Intestine	H/K ATPase, intrinsic factor	1-2 in >60-y-old
Autoimmune hepatitis		Liver	Cytochrome P450	<0.01
Autoimmune thyroiditis		Thyroid	Thyroglobulin, thyroid peroxidase	1.0-2.0
Celiac disease	60	Intestine	Transglutaminase	0.2-1.1
Graves disease		Thyroid	Thyroid-stimulating hormone receptor	0.2-1.1
Multiple sclerosis	20	Central nervous system	Myelin basic protein, proteolipid protein	0.01-0.15
Myasthenia gravis		Muscle	Acetylcholine receptor	<0.01
Pemphigus		Skin	Desmogleins	<0.01->3.0
Polymyositis		Skeletal muscle, lungs heart, joints etc.	Muscle antigens, aminoacyl-tRNA Synthetases, other nuclear antigens	<0.01
Primary biliary cirrhosis	100	Liver	2-oxoacid dehydrogenase comlpexes	<0.01
Rheumatoid arthritis	8	Joints, lungs, heart etc.	IgG, filaggrin, fibrin	0.8
Systemic lupus erythematosus	20	Skin, joints, kidneys brain, lungs, heart, etc.	Nuclear antigens (DNA, histones, ribonucleoproteins)	0.1
Type 1 diabetes	15	Pancreas (β-cells)	Insulin, GAD	0.2-0.4
Vitiligo	50	Skin	Tyrosinase, tyrosinase-related protein-2	0.4

Modified from (Marrack et al. 2001)

2.1 Genetics of autoimmune diseases

The etiology of AIDs is multifactorial with complex genetic background and environmental factors (Theofipoulos 1995; Theofipoulos 1996; Vyse and Todd 1996). Autoimmunity can be prevented on various stages of the immune response and therefore, defects in many genes can

potentially cause autoimmune disease. On the other hand, the different control mechanisms of autoimmunity often compensate for each other, and thus, a defect in one gene rarely causes autoimmunity, but such cases exist. The genetic susceptibility to AIDs is usually epistatic, and therefore various interactions between the gene products of different alleles contribute to the pathogenesis. Furthermore, the susceptibility alleles for AIDs represent often normal variants with only subtle effects on the encoded protein or its function (Morahan and Morel 2002). The genes of an individual can increase the overall reactivity of the immune system, and in this case the genetic susceptibility is usually not specific to one disease but instead, the family members susceptible to AIDs manifest many different AIDs (Eisenbarth 1999; Shamim and Miller 2000). Further, general autoimmunity susceptibility genes such as *Interleukin-2* and *CTLA-4* seem to predispose to many different kinds of AIDs (Encinas et al. 1999; Holopainen et al. 1999). In addition to affecting the overall reactivity of an individual, certain genes can specifically modify the antigen presentation/recognition in the peripheral T cell activation process or, in the selection of developing thymocytes. Class II genes in the major histocompatibility complex (MHC) region are important mediators of autoimmunity at this level. Non-MHC genes, such as the different alleles of *insulin*, can also alter antigen presentation/recognition by for example mediating the ectopic expression of insulin in the thymus and by causing susceptibility to type 1 diabetes (Vafiadis et al. 1997; Vafiadis et al. 2001). Furthermore, certain genes can directly mediate the immune response of the target tissues by affecting the physiological immune barriers, by specifically inhibiting activated lymphocytes e.g. by the production of immunosuppressive cytokines (Streilein et al. 1992) or by modifying the autoimmune inflammation (Clynes et al. 1998).

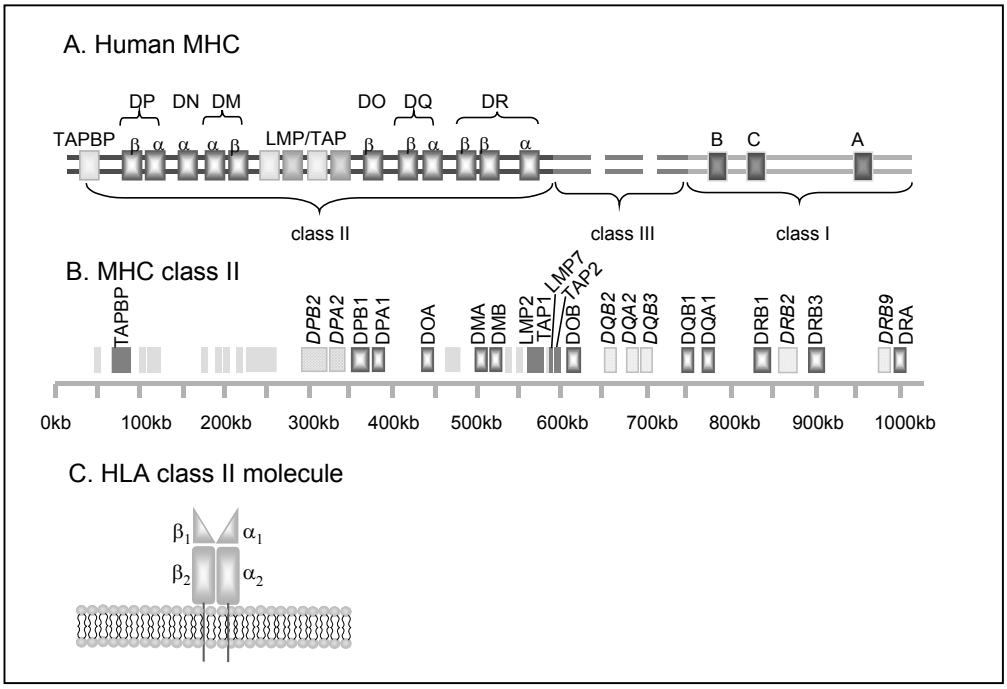
One of the most important chromosomal regions in the genetic susceptibility to AIDs is the locus for the major histocompatibility region (MHC) (in humans the HLA), which is associated with many autoimmune, immune complex and non-immunological diseases such as rheumatoid arthritis, spondyloarthropathies, multiple sclerosis, type 1 diabetes, malaria, hepatitis B and C persistence, systemic lupus erythematosus and psoriasis vulgaris (Lechler 2000). The MHC region contains over 200 genes and 40 of these genes encode the leukocyte antigens (Figure 4) (Lechler 2000). The region is highly polymorphic consisting of altogether 1556 (on 18.1.2003) different alleles. The leukocyte antigen encoding genes are divided into two functionally and structurally distinct categories: the class I and II genes. The protein products of both classes of genes function in presenting short pathogen- or self-derived peptides to T cells (Paul 1998a; McCluskey and Peh 1999; Nelson and Fremont 1999; Klein and Sato 2000b; Klein and Sato 2000a). The class II genes are the best characterized, and their role in autoimmune diseases has been clearly established.

The completion of the human (Lander et al. 2001; Venter et al. 2001) and mouse (Waterston et al. 2002) genome projects has provided us with efficient tools to identify the individual non-MHC susceptibility genes from the complex set of genes behind AIDs. The large genome-wide genetic linkage studies in humans with AIDs have been so far rather inconsistent, except for the involvement of the major histocompatibility region (Cox et al. 2001; Nerup and Pociot 2001;

Morahan and Morel 2002). Still, several well established non-MHC susceptibility alleles are now known, and these include the *IL12* gene (Ymer et al. 2002), the promoter region of the *insulin* gene (van der Auwera et al. 1993; Vafiadis et al. 1997; Vafiadis et al. 2001), polymorphisms in the *CTLA-4* gene (Kouki et al. 2000) and the *NOD2* gene (Ogura et al. 2001). Interestingly, the non-MHC susceptibility loci for the different AIDs seem to be clustered into 12 genomic regions (Wanstrat and Wakeland 2001). The clustering of the susceptibility regions has been utilised in the genome-wide analyses of animal models for AIDs, which have revealed several susceptibility loci in the syntenic mouse, rat and human chromosomal regions (Barton et al. 2001; Merriman et al. 2001). The genomic studies in congenic mice, which are genetically similar and have one isolated disease susceptibility locus, have led to the identification of

Figure 4. The major histocompatibility complex. **A**. The human MHC or HLA region on chromosome 6 contains three classes of MHC encoding genes as well as several non-MHC encoding genes. The class I and II genes encode for antigen-presenting HLA molecules. Class III genes encode for several different molecules involved in immune functions such as complement proteins 2, 4A and 4B. **B**. The genes of the MHC class II region encode for HLA molecules HLA-DR, -DQ, -DP, as well as for the peptide transporter TAP1:TAP2, for the proteasome subunits (LMP) and for the DM, DO and tapasin (TAPBP) molecules. Genes in italics represent noncoding pseudogenes. The MHC class II region accounts for the majority (620/1524) of the high number of alleles in the MHC region. **C**. The HLA-DR, -DQ and -DP molecules are heterodimers consisting of an α and a β-chain and they belong to the immunoglobulin superfamily. Each chain of the DQ and DP molecules is encoded by one functional gene. However, the α-chain of the DR molecule is encoded by one gene, whereas there are various coding genes and pseudogenes for the β-chain of the DR molecule (Paul 1998a). The domain that functions as the peptide-binding groove is encoded by β-chains and is highly polymorphic; at least 53 different DQB1 and 321 DRB1 alleles have been found (IMGT/HLA database 17/01/03, http://www.ebi.ac.uk/imgt/hla/).The polymorphisms account for the disease associations of the MHC region, as the different alleles are thought to have different antigen presenting capacities.

Modified from Immunobiology, Fifth edition (Janeway et al. 2001b)



various susceptibility genes (Boackle et al. 2001; Rozzo et al. 2001; Wanstrat and Wakeland 2001). Recently, the NOD (for non-obese diabetic) β2μMa allele was shown to be the susceptibility allele for type 1 diabetes in non-obese mice by the introduction of the susceptibility allele to the disease-resistant congenic strain (i.e. transgenic rescue). This is considered as the ”golden standard” of proof of susceptibility (Hamilton-Williams et al. 2001). Other recent approaches to find the genetic components of AIDs include (i) the genetic manipulation of mouse models by knock-out or transgenic methods, (ii) large-scale genome-wide mutagenesis of congenic mouse strains and (iii) gene arrays of the target tissues of autoimmune destruction (Morahan and Morel 2002).

2.2 Monogenic AIDs

Only a few AIDs are inherited as monogenic traits, and these include autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (discussed in later sections), autoimmune lymphoproliferative syndromes (ALPS) type I and II (OMIM 601859), and the immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) (OMIM 304790). In these rare diseases, the defective functioning of a single gene causes the autoimmune tissue destruction and the methods that allow the analysis of the molecular pathogenesis of these diseases are much more powerful than of those diseases with polygenic or complex backgrounds.

ALPS is inherited as an autosomal dominant trait (Straus et al. 1999). The phenotype components include lymphocytosis of CD4-CD8- T cells, nonmalignant lymphadenopathy, splenomegaly, hypergammaglobulinemia and autoimmune manifestations such as autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura and autoimmune neutropenia. Three different forms of ALPS with slightly different disease components, type 1a, 1b and 2, and a fourth, type III (Van Der Werff Ten Bosch et al. 2001) have been found, and different genes are responsible for each of these types. The causative gene for ALPS type 1a was identified with the help of a spontaneous animal model for the disease, the lpr mouse. The lpr mice were found not to be able to express Fas (then called Apo-1) (Watanabe-Fukunaga et al. 1992), which had earlier been found to mediate apoptosis (Trauth et al. 1989; Yonehara 1999). Next, the human homologue for Fas, the *TNFRFS6* gene, was cloned (Behrmann et al. 1994) and it was found to cause defective apoptosis in patients with ALPS type 1a (Fisher et al. 1995; Rieux-Laucat et al. 1995). The causative gene of ALPS type 1b was found to be the gene encoding for the ligand of Fas, the *FasL* gene (Wu et al. 1996). Finally, ALPS type 2 was shown to be caused by mutations in the *Caspase10* gene (Wang et al. 1999b), the gene product of which is involved in apoptotic pathways used by various different death receptors including Fas (Sprick et al. 2002).

Fas is an important regulator of T cell homeostasis, but it can also induce apoptosis in B cells, APCs and target tissues (Siegel et al. 2000). During the immune response, a negative feedback loop regulates the excess effector lymphocytes by Fas-mediated apoptosis. Consequently, defective Fas leads to massive accumulation of lymphocytes in lymphoid organs and also in a

failure to delete autoreactive naïve T cells. Fas is a member of the tumor necrosis factor receptor superfamily, and contains three extracellular region cysteine-repeat domains and an intracellular death domain, which recruits cytosolic signalling molecules. When bound to ligands, Fas forms a homotrimer on the cell membrane. Fas-mediated death is promoted by the ligation of Fas to FasL, which leads to the intracellular recruitment of Fas-associated death-domain-containing protein (FADD) and pro-caspase-8. These form the death-inducing signalling complex (DISC) (Kischkel et al. 1995). After DISC formation, pro-caspase is autocatalytically cleaved into caspase-8. The caspase-8 belongs to the caspase family of cysteine-proteases, and caspase-8 activates the cascade of the other caspases. The end-result of this process is apoptosis i.e. ”programmed cell death”, including the condensation and cleavage of the nuclear chromatin, blebbing of the cell membrane, the fragmentation of the cells and packing of the fragments into membrane-bound apoptotic bodies (Nicholson and Thornberry 1997).

IPEX is a monogenic X-linked recessive syndrome (Wildin et al. 2002), also known as XLAAD (X-linked autoimmunity-allergic dysregulation syndrome). The disease locus of IPEX was first mapped to Xp11.23-Xq13.3 (Ferguson et al. 2000) and recently, the defective gene, *Foxp3*, also called *JM2*, was identified by the positional candidate approach (Chatila et al. 2000; Ferguson et al. 2000; Bennett et al. 2001). The disease is very severe and usually results in an early death. The disease components include severe allergic inflammation, autoimmune polyendocrinopathy, secretory diarrhea, hemolytic anemia and thrombocytopenia (Powell et al. 1982). Interestingly, the gene interval for a mouse model for dysregulated lymphocyte activation, Scurfy, overlapped with the syntenic human region for IPEX (Means et al. 2000). The clinical and immunological findings of the scurfy mice resemble IPEX in humans (Lyon et al. 1990) and therefore, the critical region for the mouse locus was used to narrow the chromosomal region for IPEX. A novel gene encoding for a putative transcription factor, Scurfin, belonging to the family of the fork head transcription factor, *Foxp3*, was found to be mutated in patients with IPEX. In addition to the putative DNA-binding fork head homology domain, a C2H2 zinc finger domain, a leucine zipper motif as well as a putative nuclear localisation signal are found in the Foxp3 polypeptide. The mouse homologue for *Foxp3* has also been identified, and a mutated form of this gene was found to be the cause of the disease phenotype in the scurfy mice (Brunkow et al. 2001).

The immunological studies of the scurfy mice indicate, that the Foxp3 protein may function in both central and peripheral regulation of tolerance. The scurfy mice have an increased number of CD4+ T cells, increased cytokine levels and lymphocytic infiltrations. The immune defect in the scurfy mouse can be passed on by the transfer of scurfy T cells to a T cell-deficient recipient (athymic or SCID mice) (Godfrey et al. 1991; Blair et al. 1994). However, lethally-irradiated recipient mice for scurfy bone marrow do not develop the disease which suggests, that the thymus is involved in the disease pathogenesis (Godfrey et al. 1991). Interestingly, it has been speculated that Foxp3 may control the transcription of ectopic antigens in the thymus, and in its absence, the autoreactive T cells escape to the periphery (Patel 2001). On the other

hand, the defective Scurfin causes immunological defects that resemble those caused by manipulating regulatory CD4+CD25+ T cells, and recently, it was shown that *Foxp3* is a key regulatory gene for the development of regulatory T cells (Hori et al. 2003).

2.3 Autoimmune polyendocrine syndromes

Autoimmune endocrine diseases tend to associate with each other and with other organ-specific AIDs (Riley 1992; Caillat-Zucman 1999; Anderson 2002). Three types of autoimmune polyendocrine syndromes (APS), APS 1, 2 and 3, have been characterised (Neufeld et al. 1980; Neufeld et al. 1981; Brun 1982; Obermayer-Straub and Manns 1998, Betterle et al. 2002). APS 1 is defined by the presence of two of a triad of typical disease manifestations: Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis. The molecular basis of APS1 or APECED is the subject of this thesis and will be further discussed in subsequent sections. APS 2 was first characterised as Schmidt's syndrome by M. B. Schmidt in 1926, who described two patients with non-tuberculous Addison's disease and chronic thyroid disease (Schmidt 1926; Neufeld et al. 1980; Neufeld et al. 1981; Brun 1982; Obermayer-Straub and Manns 1998). APS 2 is defined by Addison's disease, autoimmune thyroid disease and/or type 1 diabetes, but the patients are not affected by hypoparathyroidism or candidiasis. The onset of the disease occurs in adulthood and the etiology is multifactorial (Carpenter et al. 1964; Obermayer-Straub and Manns 1998; Eisenbarth 1999). The patients develop various circulating autoantibodies (Song et al. 1996). HLA-DR3 and DR4 alleles are associated with APS2 (Eisenbarth et al. 1978; Huang et al. 1996). The molecular basis of APS2 has not been characterised in detail. APS 3 is a heterogeneous disorder involving autoimmune thyroid disease together with at least one other autoimmune disorder, excluding Addison's disease. The mode of inheritance and the molecular background of APS 3 are unknown (Neufeld et al. 1981).

3 APECED

APECED was first mentioned in the literature by Thorpe in 1929 (Thorpe and Handley 1929), and since then several names, such as APS1 or Whitaker's syndrome, have been given to this disease (Leonard 1946; Whitaker et al. 1956; Neufeld et al. 1981; Ahonen 1985). APECED is, like many other rare autosomal recessive diseases, enriched in the Finnish population as part of the Finnish disease heritage (Nevanlinna 1972b; Nevanlinna 1972a; Norio et al. 1973; Norio 2000). In 1973, Norio et al. wrote that Finland "may be called the Promised Land of rare hereditary traits for three good reasons: an exceptional population structure, an advanced level of medicine at least as compared with the "primitive" population structure, and excellent church records, which serve as a reliable population register of the vast majority of the population for about the 10 last generations". The 32 autosomal recessive, two autosomal dominant, and two X-chromosomal diseases that are enriched in Finland, but rare in other countries, are together called the Finnish disease heritage, often referred to as "the staircase of Perheentupa" after Professor Jaakko Perheentupa. In the staircase, the diseases are listed in a chronological order according to the date of the first Finnish publication on the disease. Typically, the disease-causing mutations in the causative gene are thought to have arisen in a founder population, from which the disease has migrated to the main Finnish population. For this reason, the mutations among the patients are expected to be homogenic and thus, offer a great advantage for mapping the causative genes (Peltonen et al. 1999; Peltonen et al. 2000).

In addition to the Finnish population, APECED is enriched in other isolated populations such as the Iranian Jewish (Zlotogora and Shapiro 1992), and the Sardinian (Rosatelli et al. 1998). The lifetime prevalence of APECED is rather high in the Finns, Iranian Jews and Sardians (1:25 000, 1: 9 000, and 1:14 500, respectively). In addition, APECED is relatively common in Norway (Myhre et al. 2001) and Northern Italy (Betterle et al. 1998). Multiple cases have also been reported in Great Britain (Pearce et al. 1998), North America (Scott et al. 1998), Eastern and Central European countries (Cihakova et al. 2001) and Japan (Kogawa et al. 2002a).

3.1 Genetics

APECED is an autosomal recessive disease (Ahonen 1985; Vogel et al. 2002). Our group mapped the APECED locus in the Finnish families to chromosome 21q22.3 (Aaltonen et al. 1994). The homogeneity of the locus for APECED was confirmed in a multinational group of patients with APECED, indicating that the same chromosomal locus (21q22.3) is responsible for the diseases in both Finnish and foreign patients (Bjorses et al. 1996). However, the mutations in the patients with distinct origins were expected to be different based on the haplotype segregation (Bjorses et al. 1996). A novel gene, *AIRE* (for AutoImmune Regulator), causative for APECED was positionally cloned in 1997 (Consortium 1997; Nagamine et al. 1997). *AIRE* contains 11.9 kilobases of genomic DNA and 14 exons with boundaries that follow the GT-AG rule (Mount 1982). The last exon of the gene seems to overlap with the promoter region of the *PFKL* gene, which is transcribed from the same strand of DNA as *AIRE* (Levanon et al. 1995). The *AIRE* gene harbours a putative promoter upstream of the first exon, and this

contains a TATA box, a GC box, and a CpG island. The *AIRE* cDNA, that was isolated from a human adult thymus cDNA library, contains an open reading frame (ORF) with a high GC content (69%). The mutations in the *AIRE* gene identified in the patients with APECED confirmed that *AIRE* is the causative gene of APECED (Consortium 1997; Nagamine et al. 1997), reviewed in (Bjorses et al. 1998; Peterson et al. 1998a; Peterson et al. 1998b; Aaltonen and Bjorses 1999; Meriluoto et al. 2001).

3.2 Clinical phenotype

The clinical picture of the APECED disease is highly variable between the patients (Ahonen et al. 1990; Betterle et al. 1998; Perheentupa and Miettinen 1999; Myhre et al. 2001; Perheentupa 2002) and the frequencies of the different phenotype components vary from one population to another. The factors contributing to the complexity of the disease are not yet understood, but variation between the siblings from one family suggests that factors other than the *AIRE* mutations play an important role. A triad of symptoms including hypoparathyroidism, adrenocortical failure and chronic mucocutaneous candidal infections is pathognomonic to APECED disease (Ahonen et al. 1990). As indicated by the acronym APECED, the disease may manifest itself via various endocrine deficiencies, chronic mucocutaneous candidiasis and different ectodermal dystrophies. In addition to those mentioned above, the endocrinopathies include gonadal atrophy, type 1 diabetes, gastric parietal cell atrophy and hypothyroidism (Betterle et al. 1993; Ahonen et al. 1990). The second group of manifestations, mucocutaneous candidiasis, can affect the oral, ungual, oesophagial and vaginal mucosa and the nails. Autoimmune hepatitis, enamel hypoplasia, nail dystrophies, keratoconjunctivitis, vitiligo and alopecia are other typical manifestations of APECED (Wagman et al. 1987; Lukinmaa et al. 1996; Perniola et al. 1998). Several rare components of APECED have also been reported (Arvanitakis and Knouss 1973; Ahonen et al. 1990; Friedman et al. 1991; Betterle et al. 1998; Franzese et al. 1999) (Table 3).

The clinical features of the disease have been well characterised (Table 3) (Ahonen et al. 1990; Myhre et al. 2001). In the Finnish population, the majority of the patients become symptomatic by the age of five years. Candidiasis appears at the early stages of life, hypoparathyroidism between three-five years and adrenal failure at a later stage at 11-15 years. The patients usually require continuous hormone replacement therapy, calcium and vitamin D supplements and systemic antibiotics for candidal infections. Immunosuppressive therapy is used for the treatment of autoimmune hepatitis. With careful treatment the patients usually cope with the disease and their life expectancy is only slightly decreased. However, oral squamous cell carcinoma or a sudden onset of the disease by hypocalcemic or Addisonian crisis or acute hepatitis can sometimes be of a fulminant nature (Ahonen 1990).

From the clinician's point of view, three goals are important in the management of patients with APECED: (i) the diagnosis, (ii) expert follow-up and appropriate treatment and (iii) identification of new disease components. The diagnostic analysis of mutations in the *AIRE* gene, which

Table 3. The prevalences of the common disease components of APECED in Finnish patients (Ahonen et al. 1990). In addition, rare disease components reported in the literature are listed (reviewed in (Perheentupa 2002)).

DISEASE COMPONENT	Prevalence (%)
Endocrine components	
Hypoparathyroidism	79
Addison's disease	72
Ovarian failure	60*
Hypothyroidism	4
Type 1 diabetes	12
Pernicious anemia	13
Nonendocrine components	
Mucocutaneous candidiasis	100
Enamel hypoplasia	77
Vitiligo	13
Alopecia	72
Nail dystrophy	52
Malabsorption	18
Autoimmune hepatitis	12
Keratopathy	35
Rare disease components	**No of cases**
Central diabetes insipidus	5
Growth hormone deficiency	8
Adrenocorticotropin deficiency	3
Gonadotropin deficiency	2
Hyperthyroidism	3
Autoimmune hemolytic anemia	3
IgA deficiency	>18
Asplenia	18
Cholelithiasis	7
Periodic fever with rash	11
Sjögren's syndrome	20
Oral squamous cell carcinoma	8

* Calculated for post-pubertal individuals,

covers 95% of the mutations in the Finnish patients, should be considered in patients under 30 years of age with two of the typical disease components (Table 3) without any other definite explanations. All patients and their families should be given written information on the APECED disease and the possible development of new components. Certain autoantibodies (Table 4) help in predicting the development of new disease components (discussed in section 3.3) (Perheentupa 2002). Candidiasis of the mouth is diagnosed by typical white coatings and abundant growth of *Candida albicans* on culture. Esophagial candidiasis may cause dysphagia and substernal pain and in this case, esophagoscopy should be considered due to the risk of strictures. As a therapeutical approach, systemic anticandidal medication should relief symptoms of candidiasis. The Chvostek sign is good in evaluating the hypocalcemia of a patient caused by hypoparathyroidism, but a negative sign does not exclude this. Usually the determination of plasma parathormone is not necessary, and the diagnosis of hypoparathyroidism can be made by the presence of simultaneous hypocalcemia and hyperphosphatemia, if renal insufficiency is excluded. Addison's disease is usually indicated by high serum titres of P450c21 antibodies. The inability of the zona fasciculata of the adrenal cortex to produce cortisol can be tested at an early stage with a short adrenocorticotropic

hormone test, and later on, the inability is indicated by low plasma cortisol levels. The inability of the zona glomerulosa to produce renin is indicated at early stages by supranormal plasma renin activity, and at later stages by salt craving, hyponatremia and hyperkalemia (Perheentupa 2002).

3.3 Immunological findings

The autoimmune manifestations of APECED include infiltration of lymphocytes in affected organs and the presence of various circulating, antigen-specific autoantibodies (Table 5) (Ahonen 1993; Song et al. 1996; Perniola et al. 2000). The autoantibodies are clinically valuable in making the diagnosis of APECED easier and to some degree also in predicting the course of the disease. The sensitivity of the APECED diagnosis obtained by using a set of autoantibodies (P450c21, P450scc and AADC) is 89% ((Söderbergh et al.), submitted). One group of autoantibodies is targeted against hydroxylases P450c17 (Krohn et al. 1992), P450c21 (Winqvist et al. 1992; Uibo et al. 1994b) and P450scc (Uibo et al. 1994a; Winqvist et al. 1995) that catalyse those chemical reactions required for the production of steroid hormones such as aldosterone, progesterone and cortisole (Chen et al. 1996). The presence of P450c21 precedes the development of both adrenal and ovarian failure (Ahonen et al. 1987). Another group of enzymatic autoantigens includes tryptophan, tyrosine and phelylalanine hydroxylases (TPH, TH and PAH respectively) ((Ekwall et al. 1998; Ekwall et al. 2000; Hedstrand et al. 2000), which

Table 4. The autoantigens characterised in patients with APECED. The abbreviations refer to the following: H+K+ATPase = the proton pump of the gastric mucosa, scc = side chain cleaving enzyme, TPO = thyroid peroxidase, TG = thyroglobulin, ICA = islet cell antigen, GAD = glutamic acid decarboxylase, IA-2 = tyrosine phosphatase, TH = tyrosine hydroxylase, AADC = aromatic L-amino acid decarboxylase, TPH = tryptophan hydroxylase.

Disease component	
Endocrine	Autoantigen
Addison's disease	P450c21
	P450c17
	P450scc
Gonadal failure	P450scc
Hypoparathyroidism	Calcium sensing receptor
Hypothyroidism	TPO
	TG
IDDM	ICA
	GAD65, GAD 67
	IA-2
Nonendocrine	
Alopecia	TH
Autoimmune hepatitis	P4501A2
	P4502A6
	AADC
Autoimmune gastritis	H+K+-ATPase
	intrinsic factor
Malabsorption	TPH
Vitiligo	Transcription factors SOX9 & SOX10

together constitute a group of pteridine dependent hydroxylases. All enzymes of this group are involved in the catalysis of serotonin and dopamine together with aromatic L-amino acid decarboxylase (AADC), also a target autoantigen in APECED patients (Husebye et al. 1997; Ekwall et al. 2000). The gastrointestinal dysfunction in APECED patients has been shown to be associated with the presence of TPH autoantibodies. The role of TH autoantibodies is linked to the development of alopecia areata. The connection of PAH to the clinical picture of APECED is yet unresolved. Various other autoantibodies have also been found. These are targeted against e.g. GAD65 and GAD67 (Bjork et al. 1994; Velloso et al. 1994), tyrosine phosphatase IA-2 (Gylling et al. 2000), CYP1A2 (Clemente et al. 1997; Gebre-Medhin et al. 1997), CYP2A6 (Clemente et al. 1998), thyroid peroxidase (Betterle et al. 1998), thyroglobulin (Betterle et al. 1998), the proton pump of the gastric mucosa H+K+ATPase (Karlsson et al. 1988), intrinsic factor (Mirakian and Bottazzo 1994) and the calcium sensing receptor (Li et al. 1996). Recently two transcription factors, SOX9 and 10 were found to be targets for autoantibodies in APECED patients (Hedstrand et al. 2001). The autoantibodies against P4501A2 and P4502A6 associate weakly with active hepatitis and those against SOX9 and 10 with vitiligo in patients with APECED.

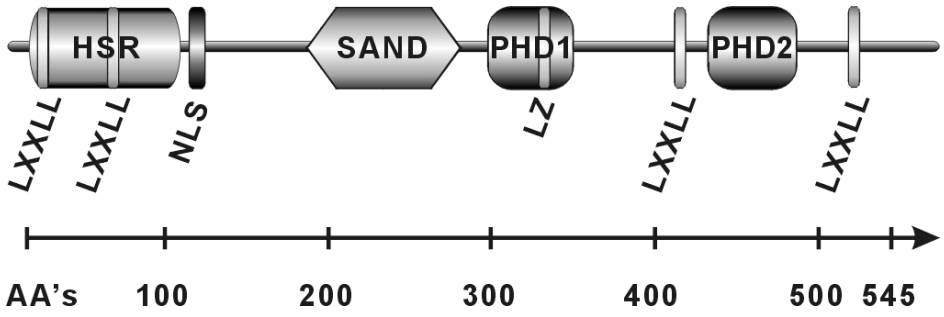
3.4 Domains of the AIRE polypeptide

The *AIRE* gene encodes a 545-amino acid protein, AIRE, with a molecular weight of 57.5 kD and a calculated pI of 7.53. The AIRE protein consists of multiple domains suggested to be involved in nuclear import, transcriptional activity, DNA-binding and homomultimerisation (Figure 5) (reviewed in (Kumar et al. 2002)). The amino terminus of AIRE harbours a domain named the HSR (homogeneously staining region) (~aa's 1-100) that according to a homology model forms a four-helix bundle structure (Pitkanen et al. 2000). HSR defines a protein family including Sp100, Sp140 (the speckled proteins 100 and 140 kD, respectively) and a putative human protein AA431918 (Sternsdorf et al. 1999). Interestingly, the homomultimerization capacity of AIRE (Pitkanen et al. 2000) (study 4 of this thesis), like that of Sp100, resides on the HSR domain (Sternsdorf et al. 1999). In the nucleus, AIRE is associated with dots that resemble the promyelotic leukemia bodies (PML) (Zhong et al. 2000), whereas in the cytoplasm, it is attached to intermediate filaments that colocalise with vimentin and α tubulin representing microtubules, and to aggregates of varying size (Bjorses et al. 1999; Heino et al. 1999a; Rinderle et al. 1999). The HSR domain of AIRE has been suggested to be responsible for the attachment of AIRE with cytoplasmic filaments (Pitkanen et al. 2001; Ramsey et al. 2002a), whereas the HSR of Sp100 is responsible for the targeting of Sp100 to the PML bodies (Sternsdorf et al. 1999).

The AIRE protein harbours a SAND domain (aa's 189-290), recently characterised as a novel DNA-binding structure (Gibson et al. 1998; Bottomley et al. 2001). The SAND domain is present in a family of proteins including members such as Sp100, AIRE, NucP41/75 and deformed epidermal autoregulatory factor-1 (DEAF-1) of *Drosophila melanogaster* (Gibson et al. 1998). The solution structure of the SAND domain of Sp100b contains a fold with five-stranded, twisted antiparallel β-sheets that pack against four α-helices (Bottomley et al. 2001).

SAND is suggested to represent a DNA-binding domain particularly characteristic for chromatin-dependent transcriptional regulation as it is often found in proteins carrying putative modules for association with chromatin, e.g. PHD (plant homeodomain type) zinc fingers. Indeed, the SAND domain of the nuclear DEAF-1 related protein (NUDR) (the human homologue for DEAF-1) can specifically bind TTCG DNA elements with a positively charged surface that contains a conserved KDWK signature (Bottomley et al. 2001). Furthermore, the homodimerization of the NUDR by a helix-loop-helix structure enhances the DNA-binding. Interestingly, the AIRE protein seems to bind to zinc finger consensus DNA sequence EGR as a homomultimer, and to oligo-TGG with high affinity when a part of a large complex (Kumar et al. 2001). Nevertheless, the specific DNA-binding capacity of the SAND domain of AIRE has not yet been shown. In addition to DNA-binding, the SAND domain of AIRE has been suggested to be necessary for the nuclear localization of AIRE (Ramsey et al. 2002a).

Figure 5. The domains of the AIRE protein. NLS= nuclear localization signal, LZ=leucine zipper.



Plant homeodomain type (PHD) zinc fingers are predominantly found in proteins that regulate transcription at the chromatin level (Aasland et al. 1995). The presence of two PHD zinc fingers or LAP (leukaemia associated protein) domains (Saha et al. 1995) in the carboxyterminal half of AIRE (aa's 296-343 and 434-475), suggests a role for AIRE in the regulation of gene expression. Indeed, AIRE has been characterised as a powerful transactivator of transcription (this thesis Study 3) (Pitkanen et al. 2000) and the PHD zinc fingers have been mapped as the transactivation domains of AIRE (this thesis Study 4) (Pitkanen et al. 2001). The PHD fingers are predominantly found in proteins that function in the regulation of transcription at the chromatin level (Aasland et al. 1995) and at present, one or more PHD fingers are found in >400 proteins (Capili et al. 2001). The characteristic motif for PHD fingers consists of seven cysteines and a histidine that are arranged in a C4HC3 consensus (Aasland et al. 1995). The solution structures of the PHD fingers of the corepressor KAP-1 and the WSTF (William's syndrome transcription factor) have been resolved (Pascual et al. 2000; Capili et al. 2001). The PHD domain of KAP-1 forms a cross-brace motif highly similar to the RING finger consisting of three β sheets, two zinc-binding sites, and an additional extended flexible region (Capili et al. 2001). The discovery of the three-dimensional structure of the PHD zinc fingers supports their

suggested role as mediators of protein-protein interactions (Capili et al. 2001). However, this function of the PHD domains of the AIRE protein has not yet been demonstrated.

In addition to the HSR, SAND and PHD domains, the AIRE protein harbours a leucine zipper motif within the first PHD domain (aa's 319-341), a putative NLS (nuclear localization signal) (aa's 113-133) and four LXXLL motifs (aa's 7-11, 63-67, 414-418, 518-524). The leucine zipper motif characterizes a major class (the other two major classes being zinc fingers and steroid hormones) of eukaryotic transcriptional regulators. In a typical leucine zipper, a leucine residue is present at every seventh position in a stretch of ~35 residues. Leucine zipper brings together a pair of DNA-binding modules by homo- or heterodimerizing the proteins of interest through the formation of an α-helical coiled coil (Stryer 1995). The leucine zipper motif of AIRE has been proposed to be involved in the homomultimerisation of AIRE (Kumar et al. 2001), but this remains to be shown. The NLS of AIRE has been shown to be functional (Pitkanen et al. 2001), but so far, the nuclear import mechanisms have not been characterised. Our recent unpublished data suggest, that the NLS of AIRE functions via the importin α-mediated mechanism (Eskelin et al., unpublished). The LXXLL domains are found in a family of nuclear proteins including CBP. In addition, they have been shown to be necessary and sufficient for the binding of proteins to ligated nuclear receptors (Heery et al. 1997). As a result of this protein-protein interaction, the proteins containing LXXLL motifs mediate the transcriptional activity of nuclear receptors. So far, no experimental data on the function of the LXXLL motifs of the AIRE protein have been presented.

3.5 *Aire* – deficient mice

Recently, two different *Aire*-deficient mouse models have been presented (Ramsey et al. 2002a Anderson et al. 2002). In both models, the mice were kept in sterile conditions, developed normally and were clinically healthy. However, autoimmune features of APECED in *Aire* -/- mice were evident. These included multiorgan lymphocytic infiltration, circulating autoantibodies and infertility.

The first *Aire*-deficient mouse model (mouse model 1) was constructed by the targeted disruption of exon 6 of the mouse *Aire* gene (Ramsey et al. 2002b). A fragment of genomic *Aire* DNA starting from intron 5 and ending to intron 6 was deleted by introducing a Neo-cassette in this position by homologous recombination. The exon 6 was chosen, as the major Finnish mutation R257X leads to a premature Stop codon in exon 6 of the human *AIRE* gene. The homologous recombination was made in J129 embryonic stem cells, and positive ES cells were injected into C57BL/6 blastocysts. The weight, size and maturation of the *Aire*-deficient mice was similar to the control mice. Interestingly, the reproduction of the *Aire*-deficient mice was abnormal, and 85% of the males and females were found to be infertile. This is in concordance with the findings in APECED patients, who develop ovarian failure in 39% of the cases at the age of 15 years, and the incidence is estimated to increase up to 72% by the age of 36 years (Perheentupa 2002). The histological analyses in 2-3 month old *Aire*-deficient mice revealed atrophy of the thymus, lymphocyte infiltrations in liver and in a single atrophied ovary, as well as

atrophy of adrenal glands were observed. The liver findings are consistent with the incidence of autoimmune hepatitis in 12% of the patients with APECED. The variation of the lymphocyte infiltrates and atrophy of the organs varied between mice, which is concordant with the highly variable phenotype of APECED patients.

The presence of autoantibodies was tested by indirect immunofluorescence stainings of frozen organ samples from healthy mice with sera from *Aire*-deficient mice. Specific staining was observed in liver, spermatogonia/spermatids, exocrine pancreas, adrenal cortex and, in a single case, in the β-cells of the islets of Langerhans. These findings are consistent with the presence of various circulating autoantibodies in patients with APECED (Table 5), but further studies are needed to show whether the autoantigens are homologous to those in humans.

The distribution of B and T lymphocytes in both thymus and periphery was analysed by flow cytometry of the thymus, spleen, lymph node and blood and by immunochemical stainings with antibodies against lymphocyte marker molecules such as (i) B220, an isoform of CD45 predominantly found in B cells, (ii) CD4, CD8, TCRβ, (iii) HSA, which is a marker for the semimature SP thymocytes in the thymus, (iv) CD62, which is downregulated in activated T cells and (v) CD44, which is expressed in effector and memory T cells. The distribution of all the studied lymphocyte populations were normal.

The responsiveness of the T lymphocytes was tested (i) using an apoptosis test in thymocyte population by annexin-V staining, which specifically binds to cells with compromised membrane phoshpolipid asymmetry, an early and specific feature of apoptotic cells. (ii) The amount of proliferating CD4+,CD8+, B220+ cells was counted by injecting bromodeoxyuridine, which incorporates into proliferating cells, intraperitoneally. (iii) The T cell population of a lymph node was stimulated *in vitro* by using CD3 antibody to promote the activation of these T cells. The activation status of the cells was analysed by using antibodies against lymphocyte markers such as CD69, which is expressed early in the activation and CD25 (the α-chain of the IL-2 receptor), which is also a general activation marker. (iv) The activation of naïve CD4+ T cells was analysed in the presence of spleen cells depleted of T cells and a mixed lymphocyte reaction. The T cells from *Aire*-deficient mice were tested against spleen cells from both *Aire*-deficient and control mice. In addition, T cells from control mice against spleen cells from *Aire*-deficient mice were tested. (v) The proliferation of lymphocytes was quantitated after immunisation of mice (Day 1 and Day 8) with hen egg lysozyme (HEL) in Freund's adjuvant.

The tests (i) and (ii) showed a normal amount of apoptotic thymocytes and proliferating T and B cells in the *Aire* -/- mice. According to test (iii), the T cells from *Aire* -/- mice follow a normal TCR signalling pathway. The test (iv) suggested that antigen presentation by APCs and tolerance induction of the T cells are normal in the *Aire*-deficient mice. Interestingly, in test (v), a hyperproliferation of *Aire*-deficient T cells was detected after the second immunisation. The complementary determining region 3 (CD3) of the TCR vβ-chain (Figure 2) of the *Aire*-deficient T cells after immunisation was analysed with spectratypa analysis. In splenic, but not in thymic

T cells, a clear alteration was found in three of the 24 Vβ families (MuBV18, MuBV19 and MuBV20). The overrepresentation of certain TCRs may cause imbalance in the T cell homeostasis, and trigger autoimmunity. In conclusion, the study by Ramsey et al. suggests that the autoimmunity in *Aire*-deficient mice may at least partially be caused by defects in the peripheral regulation mechanisms of tolerance. Further, environmental stimuli seem to trigger the autoimmune reactions in *Aire*-deficient mice, which is in concordance with the disease course in humans: the disease components of APECED manifest after birth and thus, after contact with the outside world.

A second knock-out mouse model (mouse model 2) for APECED was produced by Anderson and coworkers (Anderson et al. 2002) by the conditional targeted disruption of exon 2 including parts of the surrounding intronic sequences. The homologous recombination was made in embryonic stem cells of the Sv/129 strain, and backcrossed onto the S57/BL6 background. Thus, the defect differs from that induced by Ramsey et al. in (i) that the deletion of exon 2 affects a different functional part of the Aire protein, the HSR domain, than the deletion of exon 6 and, (ii) that the recombination was induced by the lox/cre-mediated recombination. However, aspect (i) may not have relevance, as the truncated Aire mRNA may not be translated to a protein or this protein may be instable. No truncated form of Aire could be detected in the mouse model 1, and no data concerning the truncated Aire is provided with the mouse model 2.

The general signs of autoimmunity and thus, of defective tolerance, in the *Aire*-deficient mice, were evaluated by analysing the histology of the tissue sections and the presence of serum autoantibodies. Lymphocyte infiltrations were observed in particular structures of several organs, including perivascular region of the salivary gland, ovarian follicles and retina of the eye. Serum autoantibodies against particular structures of multiple organs were also observed, including the oocytes of the ovary, parietal cells in the stomach, and the outer layer of the retina of the eye. The findings suggest, that the organs of the *Aire*-deficient mice are selectively attacked by autoimmune inflammation, and the defect in tolerance is broad, yet specific. The findings in the ovary are consistent with those in patients with APECED, but no defects in the retina or in the salivary glands have been reported in patients with APECED. Compared to the findings in mouse model 1, different organs/parts of the organs were the targets of the autoimmune attack, suggesting that the phenotype of the two models may differ to some degree. This may be explained by the (i) different type of disruption of the *Aire* gene and (ii) the genetic background that may influence the phenotype of APECED.

In order to analyse the abnormalities in the immune cells of the *Aire*-deficient mice, histological, cytofluorimetrical and functional assays were performed. Mostly, the same markers as those used in mouse model 1, and some other markers, were used. Two abnormalities were found, (i) the number of mTECs was twice as high as in the control and (ii) the number of activated/ memory T cells was doubled in the *Aire*-deficient mice. Several approaches were taken to find out which cells cause the autoimmune manifestations of the *Aire*-deficient mice. To distinguish whether Aire functions in hematopoietic or non-hematopoietic cells, four types of radiation

bone-marrow chimeras were produced, those that expressed Aire only (i) in radio-resistant cells (i.e. non-hematopoietic), (ii) in radiosensitive cells (i.e. hematopoietic cells), (iii) in neither cell type or (iv) in both cell types. Only type (ii) and (iii) chimeras exhibited autoimmune features suggesting that Aire functions in non-hematopoietic cells. Quantitative real-time PCR was performed to find out whether Aire is expressed centrally (in thymus) or peripherally. Highest expression of Aire was found in thymus, but also some secondary lymphoid organs and ovary showed expression of Aire. The expression of Aire in peripheral tissues has been a under debate, and despite of these new results, an unequivocal consensus of the tissue expression pattern of Aire is yet to be established. The authors concluded that the expression of Aire in lymphoid tissues is of particular importance.

Thymus graft experiments were performed to explore whether Aire functions in the thymus or in the periphery. The donor thymus was depleted of radiosensitive hematopoietic cells so that only the radioresistant thymic stromal cell survived, and this was transplanted to an athymic recipient $B6^{nu/nu}$. After 6 weeks, signs of autoimmunity similar to those in Aire-deficient mice were seen in those recipient mice that were transplanted by *Aire*-deficient thymus, whereas no signs of autoimmunity were present in the control mice. In conclusion, Aire seems to function in the radio-resistant stromal cells of the thymus.

To study whether the autoimmunity in *Aire*-deficient mice is conveyed by *Aire*-deficient naïve lymphocytes, these were isolated from the spleen and the lymph node of *Aire*-deficient-and control mice and transferred into alymphoid $Rag^{0/0}$ recipients. After 12 weeks, autoimmunity, similar to that in *Aire*-deficient mice, was detected in the recipient mice of *Aire*-deficient lymphocytes. This suggests, that *Aire*-deficient lymphocytes that are educated in *Aire*-deficient thymus, are sufficient to provoke autoimmunity. Further, the authors conclude that Aire expression in peripheral parenchymal tissue is not the factor controlling autoimmune attack. However, the experimental design does not directly address the question, whether Aire in the peripheral tissues regulates the autoimmune attack but rather the question of whether the Aire-deficient lymphocytes are sufficient to induce autoimmunity.

On the basis of the expression pattern of Aire in the mTECs together with its probable role as a transcriptional regulator, an intriguing hypothesis, that Aire may control autoimmunity by regulating the expression of ectopic antigens in thymic medullary epithelial cells, was proposed and tested. The lymphocyte marker expression characteristic for antigen presentation, and the number of mTECs in *Aire*-deficient mice was analysed. The mTECs were found to be overrepresented and to display normal levels of marker expression. To analyse the transcriptional profiles of the mTECs, RNA was prepared from *Aire*-deficient and control mTECs. Labelled cRNA probes were prepared from twice-amplified RNA, and hybridised onto Affymetrix chips containing cDNA fragments from 12000 mouse genes. In a large number of genes, downregulation, but not upregulation, was observed in the *Aire*-deficient mice. Thus, Aire was concluded to be a transcriptional activator of 100-300 genes that were represented on the chip, and an estimated total number of 200-1200 genes was suggested to be regulated by

Aire. To test whether the downregulated genes represented those ectopically expressed by mTECs, the previously established ectopically expressed genes (Derbinski 2001) were analysed from the array data and most of these genes were found to be silenced/repressed. The 30 most strongly down-regulated genes were analysed further, and all of these except for one, were found to be genes encoding for tissue-restricted antigens expressed ectopically. Interestingly, the analysis of all the downregulated genes showed that a significant number of the genes encoding for tissue restricted antigens were from the target tissues of autoimmune attack in *Aire*-deficient mice, such as salivary proteins 1 and 2 and zona pellucida gycoprotein 3. In addition, cytochrome P450 1A2, which is an autoantigen in patients with APECED, was found to be down-regulated. To verify these very interesting and novel findings, quantitative real-time and semiquantitative RT-PCR were performed to amplify ectopically expressed antigens from cDNA prepared from sorted mTECs. Decreased/absent cDNA encoding for most of the tested genes was found. However, not all the tested ectopically expressed antigens were depressed, suggesting that Aire regulates the expression of only a fraction of these .

In conclusion, both *Aire*-deficient mouse models seem to provide good murine models for organ-specific autoimmunity, although none of the most common phenotype components of APECED, i.e. mucocutaneous candidiasis, adrenocortical failure and hypoparathyroidism were manifested in these mice, and the mice did not have any symptoms of a disease, except for the infertility in mouse model 1. However, future studies will show whether the ”APECED mice” develop a more severe phenotype after being predisposed to different pathogens and to other environmental stimuli. Intriguingly, the *Aire*-deficient mice seem to provide extremely interesting models for the general studies of immunological tolerance and its breakdown.

AIMS OF THE PRESENT STUDY

APECED provides a unique model for the molecular studies of autoimmune diseases due to the monogenic inheritance of the disease. The following specific aims were addressed in this study:

- To collect a large multinational sample of APECED patients and analyze the possible genotype phenotype associations and HLA genes as modifying factors of the disease phenotype
- To clone the cDNA of the murine homologue of *AIRE*, the m*Aire*, and study its expression in transfected cell lines as well as in mouse tissues both at transcript and protein level
- To analyse mutations found in patients with APECED and their consequences to targeting and function of AIRE
- To characterise the *in vitro* functional roles of the different domains of the AIRE protein in subcellular localisation, homomultimerisation and transcriptional transactivation
- To study the complex formation of the AIRE protein

MATERIALS AND METHODS

The details of the several methods used in this study are described in the original publications (I-IV) according to the table below.

Table 5. Materials and methods used in this study.

Material or method	Original publication
Patients	I,III
DNA extraction	I,III
Autoantibody analysis	I
HLA allele typing	I
Mutation analysis	I,III
cDNA library screening	II
Plasmid constructs and *in vitro* mutagenesis	II,III,IV
RNA preparation	II
Detection of mRNA by RT-PCR	II
Detection of mRNA by *in situ* hybridization	II
Cell culture, metabolic labelling	II,III,IV
Transfections	II,III,IV
Raising of antibodies	II,III,IV
Immunoprecipitation	III
Protein production and purification in *E.Coli*	III,IV
Protein production by *in vitro* transcription and translation	II,IV
Protein detection by direct and indirect immunofluorescence	II,III,IV
Protein detection by immunohistochemistry	II
Protein detection by Western analysis	II,III,IV
Reporter gene assays	III,IV
Mammalian two-hybrid assay	IV
In vitro protein-protein interactions (GST pull-down)	IV
Analysis of AIRE-containing complexes	IV
Statistical analyses	I
Three-dimensional homology modelling	IV

RESULTS AND DISCUSSION

1. APECED phenotype (1)

The clinical picture of the APECED disease is highly variable even between the siblings of one family and the factors contributing to the complexity of the disease are not well understood. We collected a group of patients with APECED consisting of index patients from 104 families and 12 countries. The Finnish cohort of APECED patients was the largest, but it contained too few patients to detect possible weak genetic associations and thus, we included patients with various origins. The three most common phenotype components were mucocutanous candidiasis, hypoparathyroidism and Addison's disease. The sequence of the disease occurrence was similar to that described before, i.e. mucocutaneous candidiasis and hypoparathyroidism appeared first at mean age of 4.6 years (50/102) for candidiasis and 6.3 years (42/102) for hypoparathyroidism and the Addison's disease manifested only later at mean age of 8.9 years (15/102). There was a slight female preponderance of 54,5% vs. 45,5%.

2 Genetic factors determining the phenotype of APECED (1,3)

In several monogenic diseases, the phenotype may be modified by genes other than the disease gene and most autoimmune diseases have complex genetic etiology. The clinical phenotype of the APECED patients varies between the siblings carrying the same mutations in the *AIRE* gene. These findings imply that genetic complexity may lie behind the APECED phenotype. In this thesis, the association of the *AIRE* genotype to the APECED phenotype, as well as HLA genes as modifying genes of the phenotype of APECED, were studied.

2.1 Mutations in patients with APECED

To date, 48 mutations have been characterized in the *AIRE* gene of patients with APECED (Table 6). The mutations vary from single nucleotide substitutions, small insertions and deletions, splicing site donor or acceptor mutations to gross deletions. In the study 3, altogether 112 patients with APECED were analysed to find mutations in the *AIRE* gene. First, single strand conformation polymorphism analysis was performed on all the 112 patients. All the DNA fragments with electrophoretic shifts were further analysed by solid-phase sequencing. In 23 of the patients, only change in one allele or no change at all was observed. All the 14 exons and exon-intron boundaries of the *AIRE* gene of these patients were analysed with DNA sequencing. Altogether 16 different mutations were found from the APECED families in work 3 (Table 6). In the study 1, the coding region and exon-intron boundaries of 38 novel patients were analysed by DNA sequencing. Altogether 13 different mutations were found in 104 APECED families (Table 6). Altogether nine of the mutations in the studies 1 and 3, were novel.

Table 6. Mutations reported in the *AIRE* gene of patients with APECED by 12/2002. **Bold type** indicates that the mutation was found in our patients, star indicates that the mutation was first reported in the work 1 or 3 of this thesis.

No	cDNA change AIRE ORF	Effect on coding sequence	Affected Region of AIRE	Reference
1	30-52dup23bp	R15fsx19	HSR	(Cihakova et al. 2001)
2	43C>T	R15C	HSR	(Sato et al. 2002)
3	44G>T	R15L	HSR	(Pearce et al. 1998)
4	47C>T	T16M	HSR	(Cihakova et al. 2001)
5*	**62C>T**	**A21V**	**HSR**	**This thesis**
6	83T>C	L28P	HSR	(Pearce et al. 1998), (Heino et al. 1999b)
7	86T>C	L29P	HSR	(Kogawa et al. 2002a)
8	IVS1_IVS4	del exons 2-4	Intron 1	(Cihakova et al. 2001)
9	232T>A	W78R	HSR	(Cihakova et al. 2001)
10*	**238G>T**	**V80L**	**HSR**	**This thesis**
11	247A>G	K83E	HSR	(Nagamine et al. 1997)
12*	**254A>G**	**Y85C**	**HSR**	**This thesis**
13	269A>G	Y90C	HSR	(Pearce et al. 1998)
14	191-226del36bp	del64-75&D76Y	HSR	(Heino et al. 1999b)
15	208^209insCAGG	D70fsX216	HSR	(Heino et al. 1999b)
16	278T>G	L93R	HSR	(Ward et al. 1999)
17	415C>T	R139X	HSR	(Rosatelli et al. 1998), (Cihakova et al. 2001)
18	IVS3+2T	GT>GC	Intron 3	(Wang et al. 1998)
19*	**508^509ins13bp**	**A170fsX219**	**before SAND**	**This thesis**
20	**517C>T**	**Q173X**	**before SAND**	(Heino et al. 1999b)**, This thesis**
21	**607C>T**	**R203X**	**SAND**	(Scott et al. 1998), **This thesis**
22	682T>G	G228W	SAND	(Cetani et al. 2001)
23	755C>T	P252L	SAND	(Meloni et al. 2002)
24	**769C>T**	**R257X**	**SAND**	(Nagamine et al. 1997, Consortium 1997, Scott et al. 1998, Wang et al. 1998, Heino et al. 1999b, Ward et al. 1999), **This thesis**
25	901G>A	V301M	PHD1	(Söderbergh et al. 2000)
26*	**932G>A**	**C311Y**	**PHD1**	**This thesis**
27*	**931delT**	**C311fsX376**	**PHD1**	**This thesis**
28	**967-979del13bp**	**C322fsX372**	**LZ**	(Consortium 1997; Pearce et al. 1998; Rosatelli et al. 1998; Scott et al. 1998; Heino et al. 1999b; Ward et al. 1999; Cihakova et al. 2001), **This thesis**
29	**969^970insCCTG**	**L323fsX372**	**LZ**	(Scott et al. 1998), **This thesis**
30*	**977C<A**	**P326Q**	**LZ**	**This thesis ,** (Saugier-Veber et al. 2001)
31	1072C>T	Q358X	PRR	(Meloni et al. 2002)
32	1103^1104insC	P370fsX370	PRR	(Ishii et al. 2000)
33	**1163^1164insA**	**M388fsX422**	**PRR**	**This thesis**, (Consortium 1997)
34*	**1189delC**	**L397fsX478**	**PRR**	**This thesis**
35	**1193delC**	**P398fsX478**	**PRR**	(Consortium 1997), **This thesis**
36*	**1244^1245insC**	**L417fsX422**	**LXXLL**	**This thesis**
37	**1242^1243insA**	**H415fsX422**	**LXXLL**	(Myhre et al. 1998), **This thesis**
38	1249delC	L417fsX478	LXXLL	(Pearce et al. 1998)
39	1264delC	P422fsX478	PRR	(Heino et al. 1999b)
40	IVS9-1G>C	AG>AC	skip exon 10	(Heino et al. 2001)
41	IVS9-1G>A	AG>AA	skip exon 10	(Heino et al. 1999b)
42	1295insAC	C434fsX479	PHD2	(Wang et al. 1998)
43	1296delGinsAC	R433fsX502	PHD2	(Heino et al. 1999b)
44	1344delCinsTT	C449fsX502	PHD2	(Heino et al. 2001)
45	IVS11+1G>A	GT>AT,X476	PHD2	(Heino et al. 2001)
46	1513delG	A502fsX519	C-terminus	(Ishii et al. 2000)
47	1616C>T	P539L	C-terminus	(Meloni et al. 2002)
48	**1638A>T**	**X546C+59aa**	**STOP codon**	(Scott et al. 1998), **This thesis**

The mutations identified in the studies 1 and 3 affected all the different predicted domains of the AIRE protein. In the Finnish population, the most common mutation was a C-to-T transition at the nucleotide position 769 within exon six. This mutation changes arginine into premature STOP codon (R257X), and is predicted to lead to a truncated 256-residue protein lacking the carboxyterminal part of the AIRE protein. We observed the R257X mutation in 89% of the Finnish patients with APECED. The high prevalence of R257X as well as the haplotype segregation analyses prove this to be the founder mutation of APECED in the Finnish population. The R257X mutation is also the most common mutation worldwide, and was found in 33% of the non-Finnish patients. The haplotype analyses showed that the mutation had occurred independently six times, and this finding has been further confirmed by others (Scott et al. 1998). Thus, in different populations the R257X mutation has independent origins, which is in concordance with the common incidence of C-to-T transitions in CpG dinucleotides, in particular in arginine codons (Youssoufian et al. 1986).

The Iranian Jewish patients with APECED were also found to share a common mutation with a common haplotype, indicating a founder effect in this population. This mutation was an A- to-G transition at nucleotide position 254, predicted to change the amino acid tyrosine at position 85, to cysteine and disturb the HSR domain of the AIRE protein. Interestingly, in addition to the different type of mutation in the *AIRE* gene, the Iranian Jewish patients also manifest slightly different phenotype components of APECED than the patients from other ethnic groups. In particular, candidiasis, ectodermal dystrophies and Addison's disease are less common in the Iranian Jewish patients.

Several other types of mutations were also found, which are useful in confirming the diagnosis of APECED in patients and provide important information about the function of the AIRE protein, as they inhibit the functions of the protein. In general, nonsense mutations have been described to undergo nonsense mediated mRNA decay resulting in the total absence of the protein (Frischmeyer and Dietz 1999). Most APECED-causing mutations lead to a premature termination codon and are predicted to delete the carboxyterminus of AIRE. However, the expression of AIRE in patient cells has not been studied due to the restricted expression pattern. Thus, it remains unknown whether truncated AIRE is present in the cells of patients with nonsense mutations. The patient missense mutations that change an amino acid, indicate the essential role of this amino acid for the proper structure and function of a protein. A large proportion of the APECED-causing mutations represent missense mutations and are found in each of the predicted domains, the HSR, SAND, PHD and leucine zipper domains of AIRE. In particular, missense mutations are concentrated in the HSR domain of AIRE, suggesting a high sensitivity of this domain to structural changes.

2.2 *AIRE* genotype – APECED phenotype associations

In order to analyse the associations between the *AIRE* genotype and the APECED phenotype, clinical data from all the 104 index patients was collected from the Finnish, Swedish, Norwegian and Italian patients by one physician in each country (Drs J. Perheentupa, F. Rorsman, A-G.

Myhre and G. Weber, respectively) and from the other patients by individual physicians. Among the 104 index patient studied in work 1, an association between the absence of R257X mutation in *AIRE* and decreased frequency of mucocutaneous candidiasis was established ($p<0.001$). This needs further confirmation, as the differences in clinical observation may cause artefacts. As described in previous chapter, R257X is a nonsense mutation which either causes a carboxyterminally truncated AIRE protein or leads to the nonsense-mediated mRNA decay and a total loss of function. The mutational analysis of the AIRE protein in studies 3 and 4 may provide some explanations for the genotype-phenotype association, although the reason for the sensitivity of APECED patients to mucocutaneous candidiasis is not understood. The R257X mutation was found to severely disturb the subcellular localisation of the mutant AIRE protein, inhibit the transactivation function and complex formation of the AIRE protein, but exert almost no effect on the homomultimerization capacity of the mutant protein. Compared to the consequences of many other mutations, the R257X results in a total loss of function, whereas the less dramatic truncations of the AIRE protein and many missense mutations, especially the predicted surface mutations of the HSR domain and the mutations in the leucine zipper domain, seem to have less severe effects on the function of the AIRE protein. More extensive exploration of the phenotype-genotype associations would necessitate analysis of a larger group of patients with the rare mutation types. However, a large proportion of the patients from nonfounder populations are compound heterozygotes, and therefore, the genotype-phenotype associations may be difficult to assess. In addition, the effects of the mutations *in vivo* may be complicated to predict due to the homomultimerisation of AIRE. Moreover, the possible impact of environmental factors should be better understood and taken into account. In conclusion, despite the observed phenotype-genotype association, it seems evident that the allelic heterogeneity of the *AIRE* gene explains very little of the interfamilial variation of the phenotype.

2.3 Modification of APECED phenotype by HLA genes

The wide variation between the phenotype of patients with APECED suggests that factors other than the diversity of mutations in the *AIRE* gene affect the phenotype. Genetic complexity seems to underlie monogenic diseases, and although the diseases are inherited as monogenic traits, the clinical phenotype of these diseases may be modified by other genes (Estivill 1996; Houlston and Tomlinson 1998; Weatherall 2000). An example of ”simplex” mode of inheritance is the meconium ileus in cystic fibrosis patients, which is associated to locus 19q13 (Zielenski et al. 1999). On the other hand, the etiology of all the most common autoimmune diseases is multifactorial with complex genetic inheritance and environmental factors (Theofipoulos 1995; Theofipoulos 1996; Vyse and Todd 1996; Caillat-Zucman 1999).

The linkage and association between the HLA genes and the APECED phenotype have been studied earlier, but no significant associations have been found (Maclaren and Riley 1986; Ahonen et al. 1988; Aaltonen et al. 1993; Huang et al. 1996; Betterle et al. 1998). The negative findings in the association studies may be explained (i) by the small study groups ($n<32$) , (ii) by the serological determination of the HLA alleles, which is a less accurate method than the modern DNA-based methods or, (iii) by the fact that the HLA alleles do not modify the APECED

phenotype. Many of the disease components of APECED are associated with specific HLA alleles when appearing as isolated diseases or as a part of polyglandular syndrome type II. Considering the wide phenotypic spectrum of APECED, it is very likely that genetic complexity also exists in APECED. Further, as HLA genes play significant role in the autoimmune components of APECED in non-APECED patients, the HLA genes remain primary candidates for modifying the APECED phenotype. To better understand the genetic determinants of the phenotype variability of APECED, we analysed the genotype-phenotype associations of APECED in a group of 104 index patients from 12 different countries. The HLA-DRB1, -DQB1 and DQA1 alleles as well as the coding region and the exon-intron boundaries of the *AIRE* gene were analysed from all the patients and their siblings.

We found that the individual HLA class II alleles modify the APECED phenotype. The most definite associations to the HLA were found for alopecia, Addison's disease and type 1 diabetes. These same associations have been established earlier for non-APECED patients indicating a significant dependence of these disease components on the HLA type (Maclaren and Riley 1986; Weetman et al. 1991; Colombe et al. 1999; Yu et al. 1999) (Table 7). Alopecia was associated strongly with the DRB1* 04 allele, which is also associated with severe forms of idiopathic alopecia. However, the severity of alopecia was not determined in this study, and therefore the alopecia of our patients varied from patchy to universal. Increased risk for Addison's disease was associated with the DRB1*03 allele, which together with the DRB1*04 allele has repeatedly been found to associate with Addison's disease in non-APECED patients. The haplotype DRB1*1501-DQB1*0602 was associated with protection from type 1 diabetes. Interestingly, this is the major protective haplotype for type 1 diabetes in non-APECED patients. However, no predisposing alleles were found to associate with diabetes in patients with APECED, which may be explained by the small number of individuals with diabetes (n=13). Various other associations were also detected, but these are not supported by prior data and thus, their significance remains to be confirmed by additional studies.

The HLA associations in APECED may connect the underlying pathogenic mechanisms with those of non-APECED Addison's disease, alopecia areata and type 1 diabetes. However, although some associations connect the components of APECED with the HLA polymorphisms, many common susceptibility alleles seem not to influence the APECED phenotype. This may be explained (i) by the relatively small number of patients included in the study and by the consequent low power for establishing or excluding associations and (ii) by the fact that the different disease forms have certain aspects in common, but major differences also exist in the pathogenic pathways. An important observation is that the associations between HLA alleles and components of APECED are relatively weak, whereas in the AIDs with complex inheritance they are much stronger. This may be explained (i) by the normal HLA allele distribution in the patients with APECED and (ii) by the splitting of the relatively large (n=104) study group into smaller subgroups in the analyses of certain disease components.

Table 7. HLA associations between disease components and HLA class II alleles in index patients with APECED. Bold type indicates that prior data from non-APECED patients supports the association.

Disease component	HLA DRB1* allele	p-value	HLA DQB1* allele	p-value
Addison's disease	**03**	**0.021**		
Alopecia	**04**	**<0.001**	**0302**	**0.001**
Type 1 diabetes	**15**	**0.036**	**0602**	**0.035**
Candidiasis	01	0.019	0501	0.016
Keratopathy	04	0.032		
Keratopathy	11	0.037		
Vitiligo			0301	0.032

2.4 Autoantibodies vs. HLA

A typical sign of ongoing autoimmune inflammation in patients with APECED is the presence of serum autoantibodies (Table 4). To test whether the presence of a certain HLA allele can predispose APECED patients to the formation of certain autoantibodies, we studied associations between HLA alleles and the presence of serum autoantibodies in the 60 index patients whose autoantibody data were available. Only weak associations appeared, suggesting that in APECED the HLA alleles do not have a strong influence on autoantibody formation. This finding contrasts with those of isolated diseases, in which HLA alleles are often associated with presence of autoantibodies. There are at least three possible reasons for the negative findings in this study: (i) the number of patients may have been too small, (ii) the autoantibody titres of an individual patient fluctuate with time and the information concerning the phenotype of the patients was collected at a different time point than the autoantibodies titres of the patients and, (iii) no such associations exist.

3. Expression pattern of the mouse *Aire* gene (2)

The mouse *Aire* gene encodes a 552 amino acid protein in comparison to the 545 aa's of human AIRE (Blechschmidt et al. 1999; Mittaz et al. 1999; Wang et al. 1999a). The mouse protein has 72% identity at the amino acid level to the human homologue. In addition, all the domains of the AIRE polypeptide i.e. the HSR, SAND and PHD zinc finger domains, the leucine zipper, four LXXLL motifs and the nuclear localisation signal, are conserved in the mAire. In order to provide a molecular basis for the functional studies of mAire, we analysed its expression pattern in adult mice.

3.1 Subcellular localisation of the mouse Aire protein

In order to analyse the biological similarities of the human and mouse homologues of the AIRE/Aire proteins, the subcellular localisation of the mouse Aire (mAire) protein was studied. We expressed the *Aire* cDNA in African green monkey cells (COS-1), mouse NIH3T3 cells and baby hamster kidney cells (BHK). The cells were analysed by indirect immunofluorescense and confocal microscopy. Different types of distribution patterns were found: (i) nuclear dotted

distribution excluding the nucleoli; (ii) cytoplasmic filamentous or microtubular staining, (iii) both of these two distributions in the same cell and (iv) other staining patterns including perinuclear staining and cytoplasmic aggregates. In addition, tissue sections were analysed by immunohistochemistry. Mostly nuclear dotted staining was revealed, although some cell types including the interstitial cells of the testis and the neurons in the trigeminal ganglions exhibited specific smooth cytoplasmic staining. Thus, the mAire protein had a dual nuclear and cytoplasmic subcellular distribution similar to its human counterpart. The results suggest, that the AIRE/mAire proteins are orthologues and thus, share similar biological functions. Therefore, the mouse Aire protein may be used as a model to study the pathogenesis of APECED.

3.2 Tissue expression of the mouse *Aire* gene

Unravelling the tissue expression pattern of a protein is important for understanding its function(s). In humans, the expression of the *AIRE* gene has been studied by means of immunohistochemistry and by Northern blotting hybridisation in immunologically relevant tissues. The human AIRE protein expression was found in the thymus and lymph node (Bjorses et al. 1999; Heino et al. 1999a), in the spleen and in the peripheral blood cells (Bjorses et al. 1999). The mRNA expression pattern was similar, but also appendix and fetal liver were found to express AIRE (Nagamine et al. 1997; Heino et al. 1999a).

Interestingly, various studies on the expression of the mouse *Aire* gene indicate a wider tissue expression pattern than that detected in the human tissue studies (Blechschmidt et al. 1999; Ruan et al. 1999; Heino et al. 2000; Kogawa et al. 2002b). To provide a consensus on the expression of *mAire* in different tissues and cell types, we analysed mAire expression at mRNA and protein level using multiple detection methods. We detected mAire mRNA by RT-PCR technique in all the tissues studied, i.e. thymus, spleen, lymph node, liver, kidney, testis, brain, and fetal liver. Expression of Aire in fetal kidney was observed by cloning the partial Aire cDNA from a mouse embryonic 17d kidney library. To detect mAire mRNA at the cellular level, *in situ* hybridisation with the simultaneous use of three cRNA probes that covered the majority of the *Aire* cDNA were performed. Aire mRNA expression was detected not only in the immunological tissues such as the thymus, lymph node, bone marrow and spleen, but also outside the immune system in the brain and ovary. To detect mAire protein in tissues and cells, immunohistochemical analyses of mouse tissue sections with a polyclonal antibody against a synthetic MAP peptide corresponding to amino acids 160-176 of mAire, were performed. The strongest expression was detected in the thymus, but secondary lymphoid organs, some target tissues of the autoimmune attack in patients with APECED and many other tissues showed mAire immunoreactivity (Table 8). In the thymus, the mAire protein expression was restricted to the medulla. Similarly, the Aire mRNA distribution was concentrated in the medullar region. At the cellular level, mAire protein expression in adult mouse was observed in the thymic corpuscles, the reticular epithelial cells and in a small subpopulation of medullary thymocytes. Interestingly, mAire staining was found in many different cell types ranging from epithelial cells to neurons and glial cells. To provide adequate controls for the tissue expression pattern of mAire, preimmune serum and the anti-mAire antibody preincubated with the peptide used in

immunisations or with a COS-1 cell lysate transfected with mAirein pcDNA3/empty pcDNA3 vector, were used as controls. In addition, tissue sections from *Aire*-deficient mice were stained with the same antibody. Furthermore, immunohistochemical studies in rat tissue sections were performed using a polyclonal antibody against a human AIRE polypeptide produced in *E .coli* corresponding to amino acids 1-209 of the AIRE protein. The staining pattern of the rat tissues was similar to that in the mouse tissues.

The pattern of expression of mAire in the thymus suggests a role in the negative deletion of developing thymocytes and thus, in the central regulation of tolerance. On the other hand, the expression of mAire in many immunological tissues besides the thymus, as well as in many non-immunological tissues including several of the target organs in the pathogenesis of APECED indicate that mAire functions also outside the thymus. However, our results for the expression of the mAire protein are at odds with those of an earlier study in which mAire protein expression could not be observed by immunohistochemistry in the skeletal muscle, testis, ovary, kidney, adrenal gland, lung, lymph node, spleen or liver (Heino et al. 2000). Because the expression of the *mAire* gene is restricted to certain subpopulations of cells in many of the tissues, the overall expression level is low. This, in turn, sets limits to the detection capacity of the different methods used. The conservation of the tissue antigenicity, the affinity and specificity of the antibody, and the quality of the probe for *in situ* hybridization are the variables that most probably play an important role in explaining the contradictory results concerning the expression pattern obtained by different research groups. The expression pattern of mAire outside the immune system is supported by recent findings of an independent research group (Kumar et al. 2002) and by information from public domain databases. Several mAire ESTs (expressed sequence tags) have been sequenced from the thymus, mammary gland and embryonic stem cell cDNA libraries, and many tags ascribed to the *AIRE* sequences have been found in human SAGE (Serial Analysis of Gene Expression) libraries.

The expression of the *AIRE*/*mAire* gene in the thymus has been intensively studied (Heino et al. 2000; Zuklys et al. 2000). Consistent results show that mAire is mainly expressed in the thymic epithelial cells (TEC) (Heino et al., 2000; Zuklys et al., 2000) and particularly, in a subpopulation of corticomedullary and medullary 29+ epithelial cells in the adult tissue (Zuklys et al. 2000). The normal development of T cells requires interactions between the thymocytes and stromal cells and vice versa, as described in the section 1.2.2 of the literature review. The triple negative (TN) stages (stages I-IV) of the thymocyte maturation process correlate with the development of the thymic stromal architecture. Particularly, the TN stage II/III thymocytes are essential in the induction of TEC to form distinct cortical and medullary microenvironments (Hollander et al., 1995). During embryonic development, the mAire mRNA is expressed after E14, i.e. relatively late during ontogeny. The triple negative thymocytes (TN) at stage II/III (CD44+/CD25+ and CD44-/CD25+ cells, respectively) seem to activate the expression of mAire in TEC (Zuklys et al. 2000). This suggests, that as the *mAire* gene expression depends on activation by stage TN II/III thymocytes, it also requires correct thymic stromal organisation.

Table 8. The expression of the mAire protein in different tissues as detected by immunohistochemistry.

Immune system		
Thymus	Medulla	Reticular epithelial
	Medulla	Thymic corpuscle cells
	Medulla	Medullary thymocytes
Spleen	Red pulp	Tissue macrophages
	Red pulp	Lymphocytes
	Red pulp	Reticular cells
Lymph nodes	Medulla	Lyphocytes
	Medulla	Reticular cells
Bone marrow		Megakaryocytes, Lymphoblasts, Myeloblasts
Peripheral blood		Lymphocytes, Polymorphonuclear leukocytes
		Monocytes
Urinary tract		
Kidney	Proximal and distal convoluted tubules	Epithelial cells
	Glomeruli	Podocytes
	Kidney pelvis	Transitional epithelium
Bladder	Myometrium	Smooth muscle
Genital organs		
Testes	Seminiferous tubules	Pachytene spermatocytes, round spermatids
		Peritubular cells, Sertoli cells - few
	Interstitial cells	
Epididymis		Epithelial cells
Seminal vesicle		Epithelial cells
Prostate		Epithelial cells
Ovary	Follicles	Granulosa cells, Oocytes
Uterus	Mucosa	Epithelial cells
	Secretory glands	Epithelial cells
	Myometrium	Smooth muscle
Alimentary tract		
Salivary glands	Acini	Secretory cells
	Secretory ducts	Epithelial cells
Stomach	Mucosa	Mucosal epithelial
		Parietal cells of gastric gland
Small Intestine and colon	Mucosa	Epithelial cells
Liver		Hepatocytes and Kuppfer cells
Pancreas		Exo- and endocrine cells
		Islets of Langerhans (most of the cells)
Respiratory tract		
Lung	Bronchi	Epithelial cells
	Alveolar sacks	Epithelial and alveolarcells, macrophages
Trachea	cartilage	Undifferentiated perichondrial cells
		Differentiating chondroblasts
Endocrine organs		
Adrenal gland	Zona glomerulosa	
	Medulla	Chromaffin cells
	Zona fasciculata and reticulata	
Thyroid gland	Follicles	Epithelial and parafollicular cells
Pituitary gland	Anterior&intermediate lobe	
Nervous system		
Brain	Cerebral cortex	
	Hippocampus	Neurons
	Amygdala	Glial cells
	Hypothalamus	
	Cerebellar cortex	
	Spinal cord	
	Dorsal root ganglia	
Eye		
	Retina	Ganglial cells, Bipolar nerons

Concordantly, the RelB-deficient mice, which have an irregular thymic architecture and lack medullary thymic epithelial cells (mTEC) (Burkly et al. 1995; DeKoning et al. 1997), do not express mAire (Heino et al. 2000; Zuklys et al. 2000). This may most likely be due to an absence of cell populations that are able to express mAire, or alternatively and less likely, by the transcriptional regulation of the *mAire* gene by RelB.

4. Characterisation of the domains of the AIRE/Aire polypeptides (3,4)

The AIRE/mAire proteins, essential in intact tolerance to self antigens, exhibit features typical of transcriptional regulators. In this study, the functional regions of the AIRE/mAire proteins were characterised by utilising the mutations found in patients with APECED and individual domains of mAire. In particular, many missense mutations, which are found in each of the domains of AIRE and which provide unique possibilities to reveal the functional regions of AIRE, were analysed. The roles of the individual domains in the transactivation and homomultimerization function of AIRE/mAire were analysed, as well as the effects of mutations found in patients with APECED on the subcellular localization, transactivation function, homomultimerization and complex formation of AIRE. The structural effects of the mutations were predicted using the three-dimensional homology model of the HSR and SAND domains of AIRE.

4.1 Predicted structural consequences of the mutations in the HSR and SAND domains

To predict the structural consequences of the eleven mutations found in patients with APECED that affect the HSR domain of AIRE, we used a three-dimensional homology model of the HSR domain (Pitkanen et al. 2000), which is based on the four-helix bundle structure of the proto-oncogene CBL (Meng et al. 1999). According to their location either inside or on the surface of the bundle structure, the mutations could be grouped into those that possibly cause severe structural changes (Group 1A) and into those, that may affect those predicted protein-protein interactions of the AIRE protein mediated by the HSR domain (group 2 mutations). In addition, one of the mutations, A21V, was predicted inside the bundle structure and thus, to cause structural changes, but it was less severe than the Group 1A mutations (Group 1B mutation). The Group 1A consisted of mutations L28P, L29P, W78R and L93R, and the Group 2 from mutations R15L, T16M, V80L, K83E, Y85C and Y90C.

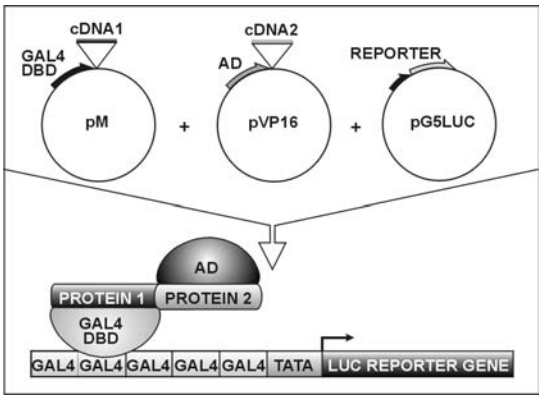
To predict the structural consequences of an APECED-causing missense mutation found in the SAND domain of AIRE with a proposed dominant mode of inheritance, and to determine whether the SAND domain of AIRE is similar to that in Sp100b, we constructed a homology model of the secondary structure of SAND on the basis of the solution structure of the SAND domain of Sp100b (Bottomley et al. 2001). Compared to the SAND domain of Sp100b, the SAND domain of AIRE had two insertions of six and seven amino acids, but the basic structure and the DNA-binding surface seemed to be conserved. The characteristic positively charged DNA-binding motif of Sp100b was formed by the amino acids KNWK, and in AIRE by the NKAR amino acids. The G228 amino acid was located on the surface of the SAND domain, and the mutation G228W found in patients with APECED, was predicted to disturb the protein-protein interactions of the SAND domain of AIRE.

4.2 Homomultimerization of AIRE

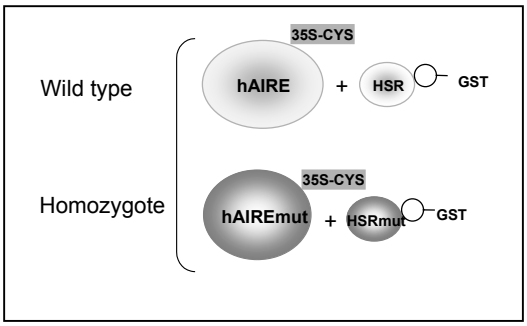
Homomultimerization may be required for specific molecular interactions, and in the case of AIRE it has been suggested to be required for DNA-binding. A phosphorylated form of the AIRE protein has been found in mono-, di and tetrameric forms *in vivo* and *in vitro* (Kumar et al. 2001). The aminoterminal HSR domain has been shown to be required for the homomultimerisation of AIRE *in vitro* (Pitkanen et al. 2000). To confirm the earlier results and to further explore the regions responsible for the homomultimerization of AIRE, we performed mammalian two-hybrid assays (Luo et al. 1997) (Figure 6A) with deletion constructs containing cDNA's encoding for individual domains of the mouse Aire protein as well as cDNA's with patient missense mutations. In order to verify the data derived from the two-hybrid experiments by an independent assay, *in vitro* GST pull-down assay (Figure 6B), was used.

Figure 6. A. The mammalian two hybrid assay. cDNAs encoding the separate domains of the mouse Aire protein as well as cDNAs encoding AIRE were cloned in a frame with the sequence encoding for the DNA-binding domain (DBD) of GAL4 and in a frame with the sequence encoding the herpes simplex virus VP16 activation domain (AD). The AIRE/Aire-GAL4 and AIRE/Aire-VP16-containing plasmids were cotransfected with a luciferase reporter plasmid in COS-1 cells to test the interaction between the proteins encoded by the inserted cDNAs. **B.** The GST pull-down assay. The wild-type AIRE cDNA encoding the HSR domain of AIRE was cloned in a frame with the sequence encoding for GST, and the cDNA encoding the full length AIRE was cloned into the mammalian expression vector pBluescript. Both constructs were mutagenised with eleven missense mutations found in patients with APECED. GST-HSR was produced in *E. coli* and full length AIRE by *in vitro* translation/transcription. The binding capacity of AIRE to GST-HSR was tested by incubating the *in vitro* translate with the GST-HSR purified from the bacterial lysate using Glutathione sepharose beads.

A



B



In the mammalian two-hybrid assay with individual domains of mAire, a homomeric interaction of mAire was detected. Furthermore, a homomeric interaction between the mHSR domains of mAire was shown. Interestingly, there seemed to be some interaction between the mSAND and mHSR domains. In the mammalian two-hybrid assay with mutated AIRE, we found that the Group 1B and Group 2 mutations of the HSR, displayed some homomultimerization capacity. By comparison, the Group 1A mutations prevented homomultimerization completely (Table 10). In the Glutathione S-transferase (GST) pull-down assay, all the missense mutations in the HSR domain of AIRE abolished the binding of the mutated HSR domain to the mutated form of *in vitro* transcribed and translated AIRE (Table 9). It is of interest, that in the mammalian two-

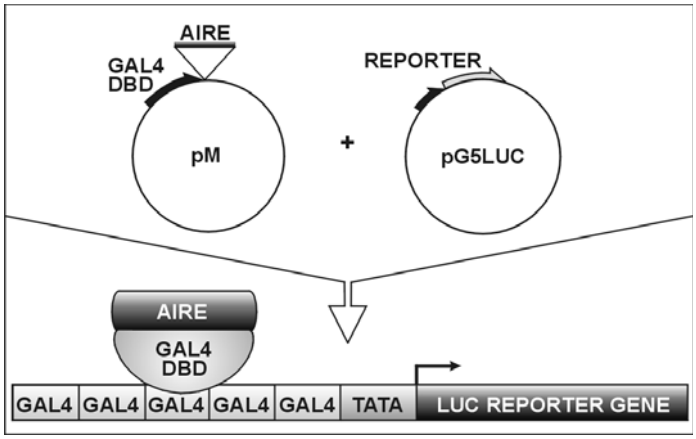
hybrid assay, the patient mutation in the SAND domain, located on the predicted surface of AIRE and which putatively disturbs the protein-protein interactions, completely inhibited the homomultimerization of AIRE. These findings suggests that the SAND domain may possess some regulatory function on the homomultimerization of AIRE. The patient mutations affecting the PHD zinc fingers (R257X, L397fsX478) and the leucine zipper (P326Q) of AIRE did not affect the homomultimerization in mammalian two-hybrid assay, although leucine zippers generally mediate the dimerization of many proteins (Figure 8). In a recent review, Kumar et al. suggest that AIRE may heterodimerize with a protein of size 32 kD (Kumar et al. 2002). The role of the leucine zipper in the possible heterodimerization of AIRE still remains to be solved.

4.3 Transactivation function of AIRE/Aire

Several predicted domains of AIRE together with its localisation in nuclear dots, suggest that this protein may play a role in the regulation of transcription. In this thesis, it was shown that the AIRE/Aire proteins act as powerful transcriptional transactivators *in vitro*. At the same time, Pitkänen et al. published similar results (Pitkanen et al. 2001). First, the wild-type AIRE and four mutations affecting the carboxyteminus of Aire and the aminoterminal HSR domain, were cloned in frame to the GAL4-DBD. The plasmids were cotransfected into COS-1 cells with luciferase reporter plasmids under two different promoters: (I) the herpes simplex virus (HSV) thymidine kinase (tk) promoter with four GAL4 binding sites (pUAS4tk-LUC) and (II) a minimal promoter containing adenovirus E1b promoter TATA sequence with five upstream GAL4 response elements (pG5LUC). The activation of the E1b promoter by wild type AIRE was significantly higher than that of the HSV-tk promoter. The results with the mutants suggested that the carboxyterminal part of AIRE is important for the transactivation capacity.

To further reveal the roles of the different regions of AIRE/mAire in the transactivation capacity, mAire cDNAs encoding for either one domain or combinations of different domains and, AIRE cDNAs carrying 13 mutations found in patients with APECED, were fused with GAL4-DBD and their transactivation capacity was tested with mammalian one-hybrid transactivation assay (Figure 7). The wild type mAire/AIRE GAL4-DBD fusion proteins activated transcription of the luciferase reporter gene 20/50-fold respectively, compared to the activity produced by a control plasmid expressing the GAL4-DBD alone. We found that the transactivation function of the mAire protein resides on the carboxyterminal part containing the mPHD zinc fingers and the leucine zipper domains, whereas isolated mHSR or mSAND domains exhibit no transactivation capacity. This confirms the results with human AIRE by Pitkänen et al. (Pitkanen et al. 2001) who used an interferon-β minimal promoter based reporter system. Further, when mSAND domain was expressed with the mPHD zinc fingers, the transactivation of AIRE decreased. The inhibitory effect of the mSAND on the mPHD finger may reflect the modulating function of the mSAND domain on transactivation. Alternatively, it may have been due to the interference of the mSAND domain with the GAL4-dependent transcription assay (but not with the interferon β reporter assay).

Figure 7. The transactivation assay. The wild type *AIRE* cDNA is fused to the sequence coding for the DNA-binding domain (DBD) of the yeast transcription factor GAL4, which also contains a nuclear targeting signal. The Aire/AIRE-GAL4 plasmids are cotransfected with a luciferase reporter plasmid carrying the adenovirus E1b promoter with five upstream GAL4-binding sites in COS-1 cells to test the transactivation capacity.



Most of the APECED-causing mutations decreased the transactivation capacity of AIRE, yet the degree of the effects varied widely (Table 10). The Group 1A mutations of the HSR domain almost completely abolished the transactivation capacity. Some of the Group 2 mutations, V80L, K83E and Y85C, led to only moderate decreases in the transactivation capacity. Somewhat surprisingly, the Y90C mutation, predicted to be located on the surface of the same α-helix as the V80L, K83E and Y85C, retained only ~30% of the wild type transcriptional transactivation activity. The two Group 2 mutations, R15L, T16M that were the most aminoterminal Group 1B mutations in the HSR domain, and the group 1B mutation A21V had only marginal effects on the transactivation capacity of AIRE. Thus, all the Group 1A mutations severely inhibited the transactivation function of AIRE, whereas the Group 1B and 2 had variable, but generally less dramatic effects. The G228W mutation in the SAND domain totally abolished the transactivation function of AIRE. Interestingly, the P326Q mutation located in the leucine zipper within the first PHD finger, which has been mapped as one of the transactivation domains, possessed ~75% of the transactivation potential compared to the wild type AIRE. In conclusion, the PHD zinc fingers seem to be the transactivating domains of mAire/AIRE, but other domains also modulate this function (Figure 8).

4.4 Association between transactivation function and localisation in nuclear dots

The AIRE-containing nuclear dots resemble PML (for promyelotic leukaemia) bodies, found in most mammalian cell nuclei (Sternsdorf et al. 1997; Hodges et al. 1998; Matera 1999). The PML bodies contain numerous proteins such as the common transactivator CBP (for CREB-binding protein), that can interact with AIRE (Pitkanen et al. 2000) and Sp100, that harbors similarly to AIRE both HSR and SAND domains. The biochemical role of the PML bodies is unclear, although various functions in cell growth control, tumour suppression, apoptosis, immune response and proteasome-mediated protein degradation have been indicated (Zhong et al. 2000). The PML bodies do not colocalize with transiently expressed AIRE in COS-1 cells,

but the colocalization cannot be ruled out without studies in the context of the cell cycle or in cells that express AIRE endogenously, as the PML bodies vary with the cell cycle (Bloch et al. 1999). The PML bodies are functionally and structurally heterogenic (Bloch et al. 1999; Muratani et al. 2002), and recently three different classes were identified in living cells; stationary bodies, those with limited localized movement and the rapidly moving metabolic-energy-dependent bodies (Muratani et al. 2002). In future, microscopy in living cells using AIRE in fusion with a fluorescent protein will provide further information about the possible localization of AIRE in PML bodies.

To study the regions regulating the subcellular localisation of AIRE, the wild type *AIRE* cDNA was cloned into the SV-Poly and pEGFP-c1 mammalian expression vectors and mutagenised with 13 mutations. African green monkey COS-1 cells were transfected with these DNA constructs, and the cells were analyzed by immunofluorescence. Most patient mutations disturbed or inhibited the transactivation capacity of AIRE, as well as the association of AIRE with nuclear dots (Table 10). The Group 1A mutations of the HSR completely inhibited the attachment of AIRE to nuclear dots. In contrast, the Group 1B and 2 mutations of the HSR domain, were mostly found to be associated with these structures. The mutation G228W, located on the surface of the SAND domain, partly inhibited the attachment of AIRE to nuclear dots and promoted the aggregation of the protein. The mutation P326Q had a minor effect on the subcellular localization of AIRE, despite the location of this mutation on the leucine zipper within the first PHD finger. This is surprising since the PHD fingers are known to be important for nuclear localization and association with nuclear dots (Bjorses et al. 2000; Ramsey et al. 2002a). Intriguingly, the degree of association with nuclear dots seems to correlate with the transactivation capacity of AIRE. Thus, our data imply that the function of AIRE as a transcriptional regulator may be connected with its presence in nuclear dots (Figure 8).

The conditional localization of AIRE either in the cytoplasm or in the nucleus may serve as a regulatory mechanism for transcriptional regulation, as has been shown for NF-kB/RelA family of transcription factors (Crepieux et al. 1997), and recently for transcription factor MIZ-1 (Ziegelbauer et al. 2001). We found that the mutations L29P, Y85C and L93R affecting the HSR, the G228W in SAND, the C311Y in the first PHD finger and the L397fsX478 causing the deletion of the second PHD zinc finger, prevented the association of AIRE with cytoplasmic filaments (Table 9). However, all the other mutant AIRE proteins were found to associate with the filaments, although less efficiently than the wild type AIRE protein. The association of the mutant AIRE proteins to the filaments did not correlate with their transactivation or homomultimerization capacity (Table 9). Earlier observations with deletion and missense mutations of AIRE suggest that homodimerization is a prerequisite for the localization in the filaments (Pitkänen et al. 2001). Our data suggest that some mutations in the HSR domain inhibit the homodimerization capacity of AIRE completely, and yet the mutants localize in the cytoplasmic filaments, thus arguing against earlier findings. It remains to be shown, whether particular regions of the HSR domain are responsible for filament binding.

Table 9. Summary of the consequences of different mutations in the AIRE protein

No	AIRE cDNA mutation	Amino acid change	Affected Domain	Nuclear dots	Cytoplasmic filaments	Trans-activation	Two-hybrid	Pull-down	>660kD complexes
	wild type			+++	+	+++	+++	+	+
1	44G>T	R15L	HSR	+++	+	++	-	-	ND
2	47C>T	T16M	HSR	+	-	++	+	-	ND
3	62C>T	A21V	HSR	++	-	++	++	-	+
4	83T>C	L28P	HSR	-	+	-	-	-	-
5	86T>C	L29P	HSR	-	-	-	-	-	-
6	232T>A	W78R	HSR	+	+	-	-	-	-
7	238G>T	V80L	HSR	+++	+	++	++	-	ND
8	247A>G	K83E	HSR	+++	+	++	+	-	ND
9	254A>G	Y85C	HSR	+++	-	+++	++	-	+
10	269A>G	Y90C	HSR	+++	+	+	-	-	ND
11	278T>G	L93R	HSR	-	-	-	-	-	-
12	682T>G	G228W	SAND	+	-	-	-	ND	+
13	769C>T	R257X	SAND	-	+	-	ND	ND	-
14	923G>A	C311Y	PHD1	+	-	+	+++	ND	+
15	977C<A	P326Q	LZ	+++	+	++	+++	ND	+
16	1189delC	L397fsX478	PHD1	-	-	-	+++	ND	+

4.5 Nuclear export of AIRE

Several of the studied mutations changed the distribution of the AIRE protein between the nucleus and cytoplasm. In particular, the Group 2 mutations of the HSR domain, all blocked the cytoplasmic localization of AIRE. Instead, the mutant proteins accumulated in nucleus, suggesting that the mutations either enhance nuclear import or inhibit nuclear export. Since the NLS of the AIRE protein seems to be sufficient for the nuclear import (Pitkänen et al. 2001) (our unpublished observations), the inhibition of the nuclear export by these mutations seems more likely. AIRE seems to be exported from the nucleus by specific nuclear export signal (NES)-dependent export, and the export signal is suggested to lie within the aminoterminus of AIRE (Pitkänen et al. 2001). Consistent with this, our data indicates that the mutations of the HSR domain belonging to Group 2 may be involved in the nuclear export of AIRE, for example by modulating the interactions of the HSR domain with complexes required for nuclear export (Figure 8) (for a review on nuclear export see (Lei and Silver 2002). Further, the G228W mutation of the SAND domain disturbed the distribution of AIRE between the nucleus and cytoplasm, and revealed excessive staining in the perinuclear region. Interestingly, the consequences of certain non-patient missense mutations in the SAND domain lead to similar perinuclear accumulation (Ramsey et al. 2002a). This type of staining may reflect defects in the nuclear transport mechanism (for a review of nuclear transport mechanisms see (Quimby and Corbett 2001) and the consequent accumulation of the mutant AIRE around the nucleus and further, suggest a role for the SAND domain in nuclear transport mechanisms.

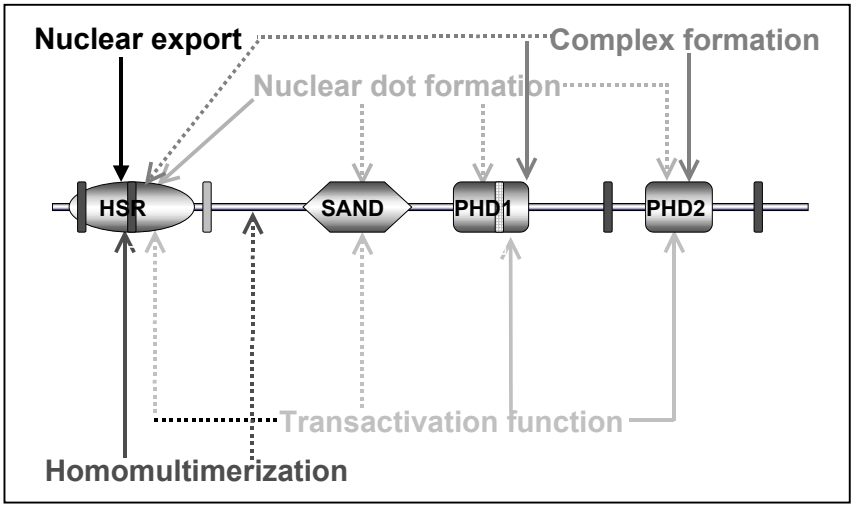
5. Complex formation of AIRE (4)

In order to find out whether AIRE occurs in homo- or heteromeric complexes, *AIRE* cDNA was expressed in transfected COS-1 cells, or alternatively, the *AIRE* cDNA and its mutagenised forms in pBluescript were translated *in vitro*. Gel filtration of the *in vitro* and cell lysates was performed under non-denaturing conditions. AIRE was detected predominantly in the void volume of molecular weight >670 kD in both the COS-1 and the reticulocyte lysates, although smaller AIRE-containing complexes were also present. It has been shown that many proteins containing PHD fingers are found in multiprotein complexes (Aasland et al. 1995) and particular protein-protein interactions carried by the PHD fingers have been reported, such as that of PHD of KAP-1 with Mi-2α, a component of the nucleosome remodelling and deacetylase complex (NURD) (Schultz et al. 2001) and that of the stromelysin-1 platelet-derived growth factor-responsive element binding protein (SPBP) with RNF4 (Lyngso et al. 2000). So far, the only specific protein-protein interaction characterised for AIRE is that with CBP. However, the specific AIRE domain(s) responsible for this interaction has not been mapped (Pitkanen et al. 2000). To further determine the parts of AIRE essential for the intermolecular interactions and formation of the AIRE containing complexes, the wild-type AIRE and selected mutants were translated *in vitro* and the lysates were analysed by gel filtration chromatography. The R257X mutation resulting in the deletion of both PHD zinc fingers by the early stop codon, resulted in the complete absence of AIRE in the large complexes. However, the disruption of the second Zn^{2+}-binding site of the first PHD finger of AIRE by the C311Y mutation (based on the structure of the KAP-1 (Capili et al. 2001) had no effect on the presence of AIRE in the high molecular weight complexes, nor the deletion of the second PHD finger by L397fsX478. Interestingly, the Group 1A mutations of the HSR domain, also disturbed the presence of AIRE in the large complexes (Table 9). Thus, our data imply that the presence of both PHD zinc finger domains and the homomultimerization of AIRE with a structurally intact HSR domain may be required for the intermolecular interactions of AIRE (Table 9, Figure 8).

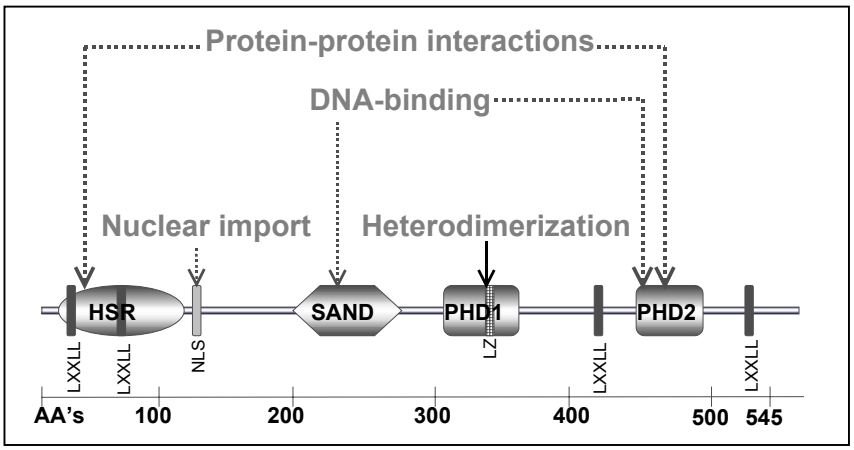
Interestingly, the predicted structural mutations of the HSR domain as well as the R257X mutation also inhibit the association of AIRE with nuclear dots, and may thus suggest that the complexes represent components of the nuclear dots. In contrast, the L397fsX478 inhibits, and the SAND and C311Y mutations significantly decrease the association of AIRE with nuclear dots, although these mutants are able to form large complexes. However, the gel filtration method separates complexes according to their size, but does not indicate whether the complexes of the wild type AIRE have the same constituents as the complexes of the mutant forms. Therefore, a correlation between the soluble complexes and nuclear dots cannot be established or excluded and needs further experiments. One possibility is that AIRE functions as a co-activator in a large transcriptional complex (for a review on transcriptional coactivators see (Naar et al. 2001)).

Figure 8. A. The functions of the different domains of AIRE that were studied in this thesis. The dotted line indicates that the corresponding function is mediated at least partly with domain. **B.** The proposed functions of the different domains of the AIRE protein.

A.



B.



CONCLUSIONS AND FUTURE REMARKS

The autoimmune attacks against restricted tissue-specific autoantigens in patients with APECED indicate that the patients have defective immunological tolerance. Because APECED is one of the rare autoimmune diseases with monogenic inheritance, it provides a unique model for the studies of more common autoimmune diseases. In this thesis, several APECED-causing mutations were identified. This has led to clinical applications in the form of a direct DNA diagnosis for APECED. Further, the sequence alterations in the *AIRE* gene may contribute to the pathogenesis of other, more common autoimmune diseases such as type 1 diabetes and Addison's disease. In future, the genetic association between the mutations and polymorphisms in the *AIRE* gene and the incidence of common autoimmune diseases should be studied in greater detail.

In general, the phenotype of any autoimmune disease is modified at many levels by genetic and environmental factors. The defective function of the *AIRE* gene may cause defects in the effector cells of the immune system and alter the immune reactivity in general. Another level of disease phenotype modification is the presentation and recognition of self antigens, which is affected by certain HLA alleles that either favour or disfavour the presentation of certain disease-associated self antigens. In this thesis, it was shown that the HLA alleles modify the phenotype of APECED patients. Interestingly, according to the present hypothesis on the function of the AIRE protein, AIRE may also function at this level of modification, by regulating the expression of ectopic antigens in thymic medullar cells, and thus, by regulating the efficient antigen presentation during the negative selection in the thymus. Many other genetic and environmental factors that may affect the phenotype of APECED are yet to be identified. The *Aire* - deficient mouse models provide us with the possibility to introduce the deficiency of *Aire* to different genetic backgrounds and thus, to explore the genetic modifying factors of the APECED phenotype. In addition, the mice can be predisposed to different environmental stimuli to identify the phenotype-modifying environmental factors. A knowledge of the factors that modify the phenotype will provide clues to the pathogenesis of APECED and better tools to predict the disease course of an individual.

The research on the tissue expression pattern of the mouse *Aire* gene, which is highly homologous to its human counterpart, provides a basis for the understanding of the function of the AIRE protein. In this thesis, mAire expression was studied using several different methods at the transcript and protein levels. The tissue expression pattern of mAire suggested that it functions in the regulation of central tolerance, but that it may also function in the secondary lymphoid organs as well as in nonlymphoid peripheral tissues. However, the different groups have obtained different types of results concerning the tissue expression pattern of mAire, and this may be due to low expression levels of mAire, which sets limits to the detection methods. An important goal in future studies is to provide an unequivocal consensus on the tissue expression of the *mAire* gene, and to reach this, alternative methods such as the construction of a transgenic mouse model with the *mAire* promoter directing the transcription of a reporter

gene, may be required. In addition, interesting future work could include the expression of mAire in embryonic tissues, as this has not been studied earlier in tissues other than the thymus. Furthermore, the detailed characterization of the expression of mAire in the secondary lymphoid tissues using expression markers, is of high importance.

The localisation of AIRE/mAire inside the nucleus as well as the conserved domains of AIRE, suggest that it may function as a transcriptional regulator. In this study, it was found that AIRE acts as a powerful transactivator *in vitro* and the regions that regulate the transactivation function of AIRE were mapped. An intriguing finding was that the transactivation function of AIRE is connected to its localization in nuclear dots, which suggests that AIRE may function as a transcriptional regulator in the AIRE-containing nuclear dots. In addition, AIRE was found to be present in a soluble form in large complexes of molecular weight over 670 kD. In this thesis, the experimental data could not confirm the association of these complexes with nuclear dots and possibly, with the function of AIRE as a transcriptional regulator. One possibility is that AIRE functions as a co-activator in a large transcriptional complex. In general, transcriptional regulation may occur at the DNA, histone, nucleosome, or chromatin level. If the soluble AIRE-containing complexes represented the transcriptional complexes/parts of this complex in which AIRE functions, the identification of the other proteins present in the complex would be a key step in revealing the mechanism of transcriptional regulation by AIRE. In addition, other approaches to identify the protein-protein interactions are important, as so far only one protein that interacts with AIRE, CBP, has been identified. In addition to protein-protein interactions, AIRE may have the capacity to bind DNA, as it harbours the SAND domain and two PHD zinc fingers, both of which are capable of binding DNA when present in other proteins. Further, preliminary data on the capacity of AIRE to bind DNA has been presented. In future, the DNA-binding capacity and the possible target sequences of AIRE need to be confirmed.

Interestingly, considering the future of the AIRE research, the two independent mouse models for APECED have for the first time provided experimental data on the function of the mAire protein *in vivo*. The mAire protein seems to function in the central regulation of tolerance, but possibly also in the peripheral regulation. According to the present hypothesis, in the absence of mAire/in the presence of defective mAire, high-affinity autoreactive T cells with TCRs specific for restricted antigens are released to the periphery as naïve T cells. However, the activation of these naïve T cells remains an open question. One possibility is that the high affinity autoreactive T cell clones in *Aire*-deficient mice may require less costimulation and a smaller amount of self antigen than the intermediate-affinity autoreactive T cell clones in a healthy individual. In addition, the *Aire*-deficient mice may have defects in their repertoire of regulatory CD25+ CD4+ T cells, as the mTECs have been suggested to be important in the selection of this population of cells. Further, the presentation of peripheral tolerising signals may be mediated by mAire, and in its absence, the peripheral tolerisation/deletion of the autoreactive naïve T cells fails. Finally, the number of autoreactive T cells may simply be too high to be regulated sufficiently, and upon incidental tissue destruction or infection/inflammation, these T cells become activated by mature APCs that present self antigens.

The recent findings concerning the function of the AIRE protein *in vitro* and *in vivo* strongly support the idea that it provides a good model for general studies of immunological tolerance and its breakdown. The variations in the promoter region of the insulin gene are associated with a decrease in the ectopic expression of insulin in the thymic medulla and interestingly, with the manifestation of type 1 diabetes. Now it seems that the thymic ectopic expression of rare antigens is controlled at least partially by mAire and in its failure, autoimmunity manifests. The autoimmune reactions in patients with APECED are mostly targeted against endocrine glands, which have highly specialised functions and which express a number of proteins that are unique for these organs, suggesting that the mechanism of ectopic expression of rare tissue antigens may have evolved to protect the highly specialised organs with specific antigens from autoimmunity. However, a role for mAire in the peripheral regulation of tolerance has also been indicated and remains to be further studied. Most importantly, the understanding of the functions of the AIRE/mAire proteins may yield valuable information on the pathogenesis of more common autoimmune diseases and provide new tools for their therapy.

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My other supervisor, Leena Palotie, is the person who interviewed me for the M.D/Ph.D program during the first year of my medical studies. At the time, I had no special understanding of genetics or molecular biology, still I was so struck by her inspiring spirit, that next summer having entered the graduate school I entered her laboratory and started my PhD project on APECED and AIRE. I specifically wish to thank Leena for the contagious inspiration, and for her devotion to science which has provided the excellent research opportunities she can now provide to young scientists at the beginning of their career.

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