Molecular characterization of the Ro52 autoantigen and its disease related epitopes

Lars Ottosson

Stockholm 2005
To my Family
ABSTRACT

The presence of high titers of autoantibodies in patient sera is characteristic for the autoimmune disorder Sjögren’s syndrome. The major targets for these autoantibodies are three intracellular proteins Ro52, Ro60 and La. It is still unclear why these proteins become targets for the immune system, and how this autoreactivity is triggered. Antibodies to the Ro52 protein have also been shown to associate with the development of congenital heart block in fetuses of anti-Ro52 positive mothers. The focus of this thesis has been to characterize the Ro52 protein at the molecular level, and to analyze the role of anti-Ro52 antibodies in congenital heart block.

By combining immunologic, biophysical and biochemical methods we have determined the protein domain composition of Ro52. The stable domains found in recombinant Ro52 correspond well with bioinformatic predictions. We have confirmed that the predicted RING-finger, B-box and coiled-coil domains are all functional in Ro52, based on secondary structure analysis and functional studies. The RING and B-box together form a single folding unit. Two Zn\(^{2+}\)-binding sites with nanomolar affinities were found within the RING-finger, while the B-box contains one independent Zn\(^{2+}\)-binding site with micromolar affinity. The region between RING and B-box appears crucial for Zn\(^{2+}\)-dependent subdomain interactions within Ro52. Secondary structure analysis of the coiled-coil domain by circular dichroism confirmed that the domain has a predominant alpha-helical fold. This domain of Ro52 was determined to consist of two structurally stable coiled-coil formations, separated by a short stretch of exposed amino acid residues. The coiled-coil domain of Ro52 forms weak homodimers, which does not exclude possible heterodimerization with an unknown interaction partner.

As a tool for studying congenital heart block, we identified and cloned two human monoclonal antibodies directed against the stretch of Ro52 recognized by antibodies associated with congenital heart block. The monoclonal antibodies were isolated from an antibody library from autoimmune patients by phage display technology. The specificities of these antibodies were fine mapped and one antibody clone was found to recognize a conformational epitope within the Ro52 coiled-coil domain. This antibody specificity was found in high frequency of sera from children affected by congenital heart block. In vitro studies with rat cardiomyocytes and in vivo studies in a rat model confirmed the importance of the certain antibody specificity in development of congenital heart block. The antibodies were found to interact with an antigen on the surface of cardiomyocytes, leading to disrupted Ca\(^{2+}\)-homeostasis in response to antibody binding. After initial increase of calcium oscillation frequency, the cells were overloaded with Ca\(^{2+}\) and died via apoptosis. This mechanism is proposed as the initiating event in congenital heart block.

In conclusion, this thesis work has revealed the presence and functionality of stable domains found in Ro52. We also suggest a mechanism for the induction of congenital heart block, and further characterized the role of specific anti-Ro52 autoantibodies in this process.

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List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Structural, functional and immunologic characterization of folded subdomains in the Ro52 protein targeted in Sjögren’s syndrome
   Submitted for publication

II. Zn\textsuperscript{2+}-binding effects on structure and folding of the Ring-Bbox-Coiled Coil (RBCC) region in Ro52, a protein targeted in Sjögren’s syndrome
   J. Hennig, L. Ottosson, C. Andrésen, M. Wahren-Herlenius, M. Sunnerhagen
   Manuscript

III. Cloning and characterization of two human Ro52-specific monoclonal autoantibodies directed towards a domain associated with congenital heart block
   Journal of Autoimmunity, 2004, 22(2), 167-77

IV. Structurally derived mutations define congenital heart block-related epitopes within the 200-239 amino acid stretch of the Ro52 protein
   Scandinavian Journal of Immunology, in press

V. Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis and mediate congenital heart block
   Journal of Experimental Medicine, 2005, 201(1), 11-17

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>AV</td>
<td>atrioventricular</td>
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<tr>
<td>BRCA1</td>
<td>breast cancer type 1 susceptibility protein</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CHB</td>
<td>congenital heart block</td>
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<tr>
<td>DA</td>
<td>dark agouti</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dsDNA</td>
<td>double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>hYRNA</td>
<td>human cytoplasmic ribonucleic acid</td>
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<tr>
<td>HLA</td>
<td>human leucocyte antigens</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-turn-helix</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>matrix-assisted laser desorption ionization mass spectroscopy</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
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<tr>
<td>NLE</td>
<td>neonatal lupus erythematosus</td>
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<tr>
<td>PML</td>
<td>promyelocytic leukemia protein</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RBCC</td>
<td>RING/B-box/coiled-coil</td>
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<tr>
<td>rfp</td>
<td>ret finger protein</td>
</tr>
<tr>
<td>RAG1</td>
<td>recombination activating gene 1</td>
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<tr>
<td>RING</td>
<td>really interesting new gene</td>
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<tr>
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<td>RING-finger protein</td>
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<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>RRM</td>
<td>ribonucleic acid recognition motif</td>
</tr>
<tr>
<td>scFV</td>
<td>single chain fragment variable</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<tr>
<td>SPRY</td>
<td>SPIa/ryanodine receptor</td>
</tr>
<tr>
<td>SS</td>
<td>Sjögren’s syndrome</td>
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<tr>
<td>TRIM</td>
<td>tripartite motif</td>
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<tr>
<td>TROVE</td>
<td>Telomerase, Ro and Vault</td>
</tr>
<tr>
<td>UnpEL</td>
<td>ubiquitous nuclear protein homolog long form</td>
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<tr>
<td>UV</td>
<td>ultra violet</td>
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INTRODUCTION AND BACKGROUND

The first part of this thesis will be a brief introduction to the concept of autoimmunity. My work has focused on molecular aspects of the autoimmune disorder Sjögren’s syndrome, especially the autoantigen Ro52, and a more thorough background will be given in these areas.

The immune system

The immune system has evolved as a way for us to defend ourselves from invading pathogenic organisms like bacteria, parasites and viruses. The immune system can be divided into the innate and the adaptive immune system, where the innate immune system is unspecific and the adaptive immune system is specific and can respond to the invading microorganism in different ways depending on the threat (Goldsby et al. 2000; Janeway et al. 2001). These two parts of the system are integrated and often interact with each other (Hoebe et al. 2004).

The innate immune system is our first line of defense against an invasion from the outside, and it can be thought of as a series of unspecific barriers that try to stop the invading organisms. The first barrier is the skin and the mucosal membrane which all microorganisms have to pass if they are to gain access into the body. The low pH in the intestine and the presence of degrading enzymes also inhibits invading pathogens. If the pathogens manage to enter the body the next barrier is phagocytic and endocytic cells and the complement system. While many cell types are capable of endocytosis (uptake of extracellular macromolecules), only specialized cells like blood monocytes, tissue macrophages and neutrophils are capable of phagocytosis (uptake of particulate material like whole microorganism). The complement system consists of serum proteins that exist in the form of inactive proenzymes. These are activated in response to different specific and non-specific immunologic mechanisms, and in their active forms may lead to direct damage to the cell membrane of the pathogenic microorganism or make them more prone to phagocytic uptake by binding to the pathogens (opsonization). Some of the inflammatory response is also a part of the innate immune system and it works by vasodilation, increase of the capillary permeability, and influx of phagocytes to the site of inflammation. The vasodilation and increased permeability of the capillaries leads to an accumulation of serum proteins that activate the complement system and increases the phagocytic uptake of pathogens.
The name “adaptive immune system” points to the major difference between this part of the immune system and the innate part: the ability to adapt to the changes in the threat from pathogenic microorganisms. There are four attributes that characterize the adaptive immune system: specificity, diversity, memory and self/nonself recognition. The specificity makes the immune system able to distinguish between different pathogen derived molecules when they differ in as little as one single amino acid (aa). When the molecules that are going to recognize different pathogen derived molecules are generated, there is an exceptional amount of possible specificities which leads to the great diversity that allows for recognition of many different pathogens.

Some of the cell types of the adaptive immune system are long lived and these cells contribute to the immunologic memory that makes it possible for the immune system to remember foreign molecules that it has experienced before. The next time the same foreign molecule is recognized again, the immune system can mount a specific response much faster. Finally, an important issue for the immune system is the ability to distinguish molecules of the body’s own tissues from foreign pathogen-derived molecules. This is called the self/nonself recognition. It is intuitive to think that the only important thing for the immune system is to defend the body from pathogenic microorganism, but not to attack the own body is of the same importance.

Two different groups of cells are necessary for the ability to generate an effective immune response; the lymphocytes and the antigen presenting cells. The lymphocytes can be of many different types and they are all produced by hematopoiesis in the bone marrow. The major groups of lymphocytes are T cells and B cells, and both these cell types are antigen specific in their mature state. T cells migrates from the bone marrow to the thymus were they mature, while B cells stay and mature within the bone marrow. The B cells then enters the circulation and the peripheral lymphoid tissues where they may encounter their specific antigens. Both B and T cells express receptors specific for just one antigen, but the receptors are of different types. There are several different types of T cells and new subgroups are still being defined. The classic types of T cells are the CD8+ cytotoxic T cells and the CD4+ T helper cells. The T helper cells function as activators of B cells and cytotoxic T cells and produce a variety of different cytokines, which are signal molecules of the immune system. The cytotoxic T cells are the effector cells that can kill infected cells.

B cells have a function as professional antigen presenting cells and they produce antibodies as well as different cytokines. The antigen specific receptor on the T cells is called T cell receptor while the antigen specific receptors on B cells are membrane bound antibodies. When a B cell binds its specific antigen on the surface bound antibodies these will be internalized, the antigen processed and later antigen derived peptides will be expressed on MHC II molecule on the surface of the B cell. When a T helper cell specific
for the presented antigen peptide recognize this MHC II bound peptide it will activate the B cell that in response will start to proliferate and differentiate to antibody producing plasma cells or memory B cells.

The antibodies produced by the B cells are secreted (or in case of the B cell receptor, membrane bound) antigen specific molecules. Each mature B cell can only produce antibodies of a single specificity and under normal conditions it needs T cell stimulation for production of antibodies. There are five different isotypes of antibodies with different function. Antibodies can bind antigens in solution or bind to whole pathogens like bacteria, and by binding they mark the antigen/pathogen for phagocytic uptake, so called opsonization. Bound antibodies can also activate the complement system.

**Autoimmunity**

An important attribute of the immune system is the ability to distinguish self from nonself. When the immune system fails with this it will generate an immune response against cells and tissues in its own body, an autoimmune reaction. In patients suffering from an autoimmune disorder, autoreactive cells of different kinds like CD4⁺ T helper cells, CD8⁺ effector T cells and B cells/plasma cells can be identified. How the transition from a normal state to an autoimmune state of the immune systems is induced is still debated. One explanation can be that an infection triggers a normal immune response that happens to be cross reactive with one or several self antigens. Another is that certain self antigens no longer are recognized as such due to modifications or failure of the presentation system. Autoreactive cells do not only exist in patients suffering from autoimmune disorders, but are commonly found in non-affected individuals too (Danke et al. 2004). Why they are kept silent in some people but not all can be due to differences in the regulation system or environmental differences that keep them from being triggered.

The developing T and B cells are kept under strong regulation and only clones that recognize foreign antigens but not self antigens are supposed to be allowed to mature. For T cells this training takes place in the thymus by both negative and positive selection (Starr et al. 2003). The T cell receptor of maturating T cells have to be tested if they can recognize the self MHC-molecules, if they do they are positively selected and will survive, while the rest will die via apoptosis. MHC class I and II are the two kinds of peptide binding molecules which the cells use to present peptides to T-cells. In humans the MHC-genes are called HLA-genes. In the next stage of maturation, called the negative selection, the T cells are tested for autoreactivity and if their T cell receptors are specific for self antigens, they will die via apoptosis. The B cells are selected in a similar
way but their selection take place in the bone marrow, (Edry and Melamed 2004). The mechanisms behind the development of an active autoimmune response are still not defined, but in some way the tolerance of self antigens is broken. What is known is that both genetic and environmental factors are associated with the induction of different autoimmune diseases (Tsao 2004) and that many autoimmune diseases are associated with certain HLA genes (Undlien et al. 2001; Gebe et al. 2002).

**Autoimmune diseases**

Autoimmune diseases are a heterogeneous group of diseases but they have the presence of autoreactive immune cells in common. The autoimmune tissue damage can be mainly cell mediated as in Multiple Sclerosis (MS) (Steinman 1996) or mainly mediated by the humoral immune response via autoreactive antibodies as in systemic lupus erythematosus (SLE) (Kotzin 1996), but most common is a combination of both. The autoimmune diseases may also be divided into organ specific and systemic diseases. An example of an organ specific autoimmune disease is insulin dependent diabetes mellitus (IDDM). In IDDM, the target organ for the autoreactive cells is the pancreas where the insulin producing β-cells are exclusively attacked (Durinovic-Bello 1998; Yoon et al. 1998). The destruction of the β-cells result in an inability for the body to produce insulin and therefore exogenous insulin has to be supplied.

In the systemic autoimmune diseases multiple organs are attacked and there is often a wider span of autoantigens. Examples of systemic autoimmune diseases are Rheumatoid arthritis (RA) and SLE, while Sjögren’s syndrome (SS) displays both organ specific and systemic manifestations.

**Sjögren’s syndrome**

The disease Sjögren’s syndrome, got its name from Henrik Sjögren, a Swedish ophthalmologist who wrote his doctoral thesis in 1933 about eye and mouth manifestations in patients with this autoimmune disorder (Sjögren 1933). Reports on the prevalence of SS varies between 0.05% and more than 4%, depending on which set of criteria has been used and also the age of the people in the analyzed group (Jacobsson et al. 1989; Manthorpe et al. 1998; Thomas et al. 1998). Sjögren’s syndrome is more common in women than in men, with a female to male ratio of about 9 to 1 and the risk of developing SS increases with age. The most commonly affected organs in SS are the exocrine glands and mainly the salivary and lacrimal glands. The most prominent
symptoms, dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca) are caused by
destruction of the mentioned glands. This tissue destruction is caused by infiltrates of
immune cells into the target organs (Figure 1). The major part of the infiltrating cells are
T cells, predominantly CD4+ cells, while up to 15% are B-cells and plasma cells (Fox et
al. 1984), and around 5% are monocytes, macrophages, dendritic cells and other cell
types.

Figure 1. Salivary gland biopsies from a healthy control and a patient with Sjögren’s
syndrome. The left picture displays a hematoxylin stained biopsy from a healthy individual. The
right picture displays cell infiltrates in a salivary gland biopsy from a Sjögren’s syndrome patient,
stained with hematoxylin. Photo: Monica Ek

One of the most characteristic serologic features of SS is the often very high titers
of autoantibodies in the patients. These autoantibodies are commonly directed against a
few certain autoantigens in all patients. The most prevalent autoantibodies are directed
against the Ro52, Ro60 or La proteins which will be discussed in detail later.
Autoantibodies directed against two antigenic particles were first detected, and these
particles were denoted SSA and SSB. Later the SSB particle was identified as the La
protein and the SSA particle was found to consist of two different proteins, Ro60 and
Ro52 (Ben-Chetrit et al. 1988; Deutscher et al. 1988; Ben-Chetrit et al. 1989). Ro60 and
La has been shown to be associated with small cytoplasmic RNA molecules, hYRNA,
forming ribonucleoproteins (Ro-RNPs) (Fabini et al. 2000). Ro52 has also been
suggested as being part of the Ro-RNP particle (Figure 2), either by direct binding to the
hYRNA or by interacting with another RNA-binding protein (Cheng et al. 1996), but it is
still unclear (Boire et al. 1995). There are also other autoantigens like α-fodrin (Haneji et
al. 1997), but compared to the Ro and La antibodies they are less common. SS patients
often have hypergammaglobulinemia and it has been shown that they have a shift in B cell population and fewer memory B cells than healthy individuals (Bohnhorst et al. 2001; Bohnhorst et al. 2002; Dörner and Lipsky 2002).

Among the numerous systemic manifestations in SS patients are renal involvement, respiratory tract involvement and vascular involvement. Patients with SS also have a higher risk of developing non-Hodgkin’s lymphomas (Kassan et al. 1978), most commonly of B cell origin. There is no absolute diagnosis of SS, instead sets of criteria have been used. Different criteria have been used in different countries in parallel due to the problems in finding a common set of criteria (Homma et al. 1986; Skopouli et al. 1986; Fox and Saito 1994; Vitali et al. 1994). Lately, joint effort in combining the different sets of criteria has produced one set that can be used worldwide (Manthorpe 2002; Vitali et al. 2002). This new set of criteria consist of six criteria of which at least four has to be fulfilled; 1) Ocular symptoms, 2) Oral symptoms, 3) Ocular signs, 4) Histopathology, 5) Salivary gland involvement and 6) Autoantibodies to Ro52, Ro60 and/or La. Of these six criteria, at least one of number 4 or 6 must be fulfilled for a Sjögren’s syndrome diagnosis.

**Figure 2. Schematic view of the Ro-RNP.** Ro60 and La are known to bind the hYRNA-molecule to form Ro-RNP particles. Ro52 has also been suggested to bind to the complex, either directly, via Ro60 or via another protein.
**Systemic lupus erythematosus**

Systemic lupus erythematosus is another heterogeneous autoimmune disease which diagnosis is based on fulfillment of a set of criteria (Tan et al. 1982). SLE has typical manifestations in terms of malar rashes, photosensitivity, renal disorders due to immunocomplexes and high titers of autoantibodies. The autoantigens targeted in patients with SLE are more diverse than for SS, but autoantibodies to DNA (double stranded or single stranded DNA) and DNA associated proteins like histones are typical but antibodies to Ro52, Ro60 and La are also common. Some of the autoantigens are targeted in many other rheumatic diseases, but the anti-dsDNA antibodies and autoantibodies directed against the Sm and the ribosomal P proteins are highly specific for SLE.

**Neonatal lupus erythematosus and congenital heart block**

Neonatal lupus erythematosus (NLE) is a disorder that can affect fetuses of mothers who are positive for Ro and La autoantibodies (Dörner et al. 2000; Brucato et al. 2002). These mothers can be asymptomatic, but many of them suffer from SS or SLE and it is common that the asymptomatic mothers develop an autoimmune disorder later on. Congenital heart block (CHB) and skin manifestations are the two most common manifestations in NLE. CHB is a potentially fatal heart disorder affecting the cardiac signal transduction system. The heart block can be graded into three major degrees of blockage (Figure 3), where atrioventricular (AV) block I is a prolongation of the PR interval, AV block II an inhibition of every second or third impulse through the AV node and an AV block III being a complete block of the electric impulses in the AV node. A third degree block may be fatal and if not, a life long use of pacemaker is usually necessary.

CHB is a passively transferred autoimmune disorder due to transplacental passage of Ro/SSA and La/SSB autoantibodies, and the association between these autoantibodies and CHB has been known for a long time (Harley et al. 1985; Lee et al. 1985; Litsey et al. 1985) (Figure 3). The mechanism that leads to the heart block is not clarified in detail, but signs of inflammation like antibody and complement deposits (Lee et al. 1987; Tran et al. 2002a) leading to fibrosis (Clancy and Buyon 2004) in the cardiac tissue has been demonstrated. It is also debated if all antibodies against Ro52, Ro60 and La are pathogenic or if certain specificities are necessary for the development of the heart block. In some reports (Boutjdir et al. 1997; Salomonsson et al. 2002), autoantibodies against Ro52 show the highest correlation with the disorder while antibodies against the La protein has been determined to be the disease associated antibody in other studies (Tran
et al. 2002a). Several studies demonstrate that heart block can be induced in animals by immunization with Ro52, Ro60 and La or by injections of affinity purified autoantibodies in animals or in animal hearts (Boutjdir et al. 1997; Miranda-Carus et al. 1998; Mazel et al. 1999; Restivo et al. 2001), which supports the view of CHB as a passively transferred autoimmune disorder.

Figure 3. AV block detected with Doppler echocardiography and an artistic view of antibody dependent congenital heart block. The left panel shows Doppler echocardiography recordings from fetuses with different degrees of AV block. The upper picture displays an AV block I, the middle picture an AV block II and the lower picture an AV block III (Recordings: Sven-Erik Sonesson. The right panel shows the concept of congenital heart block due to passive transfer of maternal autoantibodies. The picture to the right is the cover picture from the January 2005 issue of The Journal of Experimental Medicine, made by Rachel Urkowitz. The picture is reprinted with permission from The Journal of Experimental Medicine.
Autoantigens and autoantibodies in SS and SLE

A common feature of the autoimmune diseases SLE and SS is the presence of high titers of autoreactive antibodies in patient sera. The targets for these autoantibodies are different molecules, autoantigens. The autoantigens can be both proteins and nucleic acids. Among the major protein autoantigens there are nuclear, cytoplasmic and extracellular proteins. In some organ specific autoimmune diseases a pathogenic function has been determined for a certain specificity of autoantibodies. In, for example, Grave’s disease binding of antibodies directed against the thyroid stimulating hormone receptor results in blocking of the receptor followed by an excessive thyroid hormone production (de Bruin et al. 1988; Morgenthaler et al. 1996). The pathogenic role for autoantibodies in systemic autoimmune diseases is more controversial. High titers of autoantibodies can lead to accumulation of immunocomplexes and deposits in the kidneys. These deposits will lead to complement activation and will generate an inflammatory response which can cause major tissue damage and organ failure. However, the formation of immunocomplexes is a result of antibodies binding to autoantigens in solution and antibodies directed against many different autoantigens can be found in the immunocomplexes (Mannik et al. 2003). There have been many studies trying to find evidence for a pathogenic mechanism for certain autoantibody specificities. One example is for the anti-dsDNA autoantibodies common in SLE (Swissa et al. 1996; Mason et al. 2004; Putterman 2004).

Ro52, Ro60 and La are the three main autoantigens in SS. In clinical practice, these autoantigens are commonly denoted SSA (Ro52 and Ro60) and SSB (La). Ro60 is a 60 kDa protein that was identified as a part of a ribonucleoprotein complex (the Ro-RNP). Ro60 was cloned in 1988 (Deutscher et al. 1988; Ben-Chetrit et al. 1989). By sequence homology search, a TROVE-module (Bateman and Kickhoefer 2003) containing an RNA-binding motif was identified in Ro60. This RNA-binding motif is involved in the binding of Ro60 to the hYRNA molecules to form Ro-RNPs. The La protein is a 48 kDa RNA-binding protein (Chambers et al. 1988; Chan et al. 1989a; Chan et al. 1989b) that contains one RNA recognition motif (RRM) and one La helix-turn-helix domain (La HTH). La is thought to function in transcription termination of RNA polymerase III transcripts (Gottlieb and Steitz 1989; Pannone et al. 1998), and in this sense may also associate with the Ro-RNP particles (Pruijn et al. 1997; Bouffard et al. 1999).
Figure 4. Alignment of Ro52 protein from different species. The Alignment was performed by using the software at http://www.ebi.ac.uk/clustalw/. The consensus symbols are: *= identical amino acid, := conservative substitution, .= semi-conservative substitution.
The Ro52 protein

The human form of Ro52 is a 475 aa protein that was cloned in 1991 (Chan et al. 1991; Itoh et al. 1991). Since then the homologous proteins in mouse (Keech et al. 1996) and in cattle (Shusta et al. 2003) have been identified and cloned. The sequence identity between the murine and the human Ro52 proteins is 70% on the amino acid level, and the similarity is even higher in parts containing predicted functional domains (Figure 4) (Keech et al. 1996). The human Ro52 gene has been mapped to chromosome 11 in the region 11p15.5 (Bepler et al. 1999). There are several synonyms for Ro52; Tripartite motif protein 21 (TRIM21), RING-finger protein 81 (RNF 81) and 52 kDa Ro/SSA.

![Figure 5. Schematic view of predicted protein domains in Ro52. Within the coiled-coil domain the area with predicted low coiled-coil propensity (hatched) and the leucine zipper (dark grey) are visualized.](image)

**Ro52 and the TRIM-protein family**

By sequence homology studies, several protein domains can be predicted in Ro52 (Figure 5). A leucine zipper was described contained within aa 211-232 and three zinc-finger domains were predicted in the N-terminal of Ro52 (Chan et al. 1991). The three predicted zinc-fingers in Ro52 were later identified as two; one RING-finger with two Zn$^{2+}$-binding sites and one B-box with one Zn$^{2+}$-binding site. The middle part of the protein (approximately amino acid 130-260) including the leucine zipper, has a predicted high potential for a coiled-coil structure. The build-up of an N-terminal RING-finger followed by a B-box motif and a coiled-coil domain is a common combination and two different names for this family of proteins have been suggested; tripartite motif proteins (TRIM) (Reymond et al. 2001), or RING/B-box/coiled-coil (RBCC) (Reddy et al. 1992; Borden 1998) (Figure 6). The TRIM proteins have different C-terminal domains and the localization can be both nuclear and cytoplasmic (Reymond et al. 2001). The C-terminal part of Ro52 contains a predicted B30.2 or rfp-like domain also find in other TRIM-proteins (Patarca et al. 1988; Takahashi et al. 1988). The TRIM proteins have different
functions. Some of the TRIM members have been identified as transcription modulators (Shimono et al. 2000), including involvement in the ubiquitin dependent protein degradation system (Horn et al. 2004). Several TRIM proteins are also proto-oncogenes and form oncogenes after chromosomal translocation (Grignani et al. 1994; Le Douarin et al. 1995).

![Schematic view over some members of the TRIM-protein family.](image)

**The RING-finger**

The most studied of the two predicted Zn\(^{2+}\)-binding motifs in Ro52 is the RING-finger. The name is derived from Really Interesting New Gene, and the first RING-finger was identified in 1993 (Freemont 1993; Lovering et al. 1993). The RING-finger motif is not only found in TRIM proteins but is a common motif in other protein families as well. From the beginning it was suggested that the RING-finger was a strictly DNA-binding motif, but later it has been associated with ubiquitination and especially as a functional domain in E3 ubiquitin ligases (Trockenbacher et al. 2001; Urano et al. 2002; Linares et al. 2003) and different kinds of protein–protein interactions (Saurin et al. 1996). At least ten RING-finger structures from different proteins have been published to date. Among them are the RING-fingers from RAG1 (Bellon et al. 1997), where the RING-finger promotes homodimer formation, the acute promyelocytic leukemia proto-oncoprotein...
(PML) (Borden et al. 1995a), and the breast cancer type 1 susceptibility protein (BRCA1) (Brzovic et al. 2001) where it promotes heterodimerization. The solved structures are very similar. The RING-finger binds two Zn$^{2+}$ ions in a cross braced motif using eight conserved cysteine or histidine residues (Barlow et al. 1994) (Figure 7A). It has been shown that the predicted RING-finger region as well as the B-box region have affinity for Zn$^{2+}$ (Pourmand and Pettersson 1998).

**The B-box**

The B-box motif is less studied than the RING-finger and has so far not been detected in proteins outside the TRIM family. The TRIM proteins have one or two B-boxes located between the RING-finger and the coiled-coil domain. If there are two B-boxes, they are of two slightly different sub types (Borden et al. 1996). So far only one B-box structure has been solved and published (Figure 7B) (Borden et al. 1993), showing that one Zn$^{2+}$ ion is bound in a tetrahedral manner by four conserved cysteine and histidine residues. While RING-fingers have been identified as the crucial domain for several different functions as ubiquitination and other protein-protein interactions, no function has still been clearly assigned to the B-box domain. However, some results indicate that the presence of a correctly folded B-box is essential for homo- and hetero-dimerization of the coiled-coil domain on the C-terminal side of the B-box (Cao et al. 1997; Cao et al. 1998).

**Figure 7. Structural view of a RING-finger and a B-box.** (A) Solution structure of the RING-finger domain from the PML protein (Borden et al. 1995a). (B) Solution structure of the B-box domain from the Xnf7 protein (Borden et al. 1995b). The residues involved in the Zn$^{2+}$-binding are highlighted. The structural views were obtained by using the RasTop/RasMol 2.6 software.
The coiled-coil domain and the leucine zipper

As in other TRIM proteins, the middle part of Ro52 comprises a coiled-coil domain (Reymond et al. 2001). In Ro52 the predicted coiled-coil domain spans most of the amino acid sequence from the end of the B-box to approximately aa 250 with just a short sequence of low coiled-coil probability between aa 167-180. In the coiled-coil domain a leucine zipper (Alber 1992) is situated with the first leucine of four at aa 211 and the last at aa 232. The leucine zipper was identified as an essential dimerizing motif in some DNA-binding proteins, but was later found in different kinds of proteins as well. The coiled-coil domain is a very common protein motif and was first described in fibrous proteins, but was later found to be one of the most common motifs for multimerization in other proteins as well (Beck and Brodsky 1998). Several coiled-coil based multimerizations have now been studied in molecular detail (Burkhard et al. 2000; Revington et al. 2002) and it has been shown that coiled-coil domains can promote hom- and hetero-dimerization (Paris et al. 2003) as well as multimers with several alpha helices (Cohen and Parry 1990; Harbury et al. 1993) and some of the TRIM proteins are known to form homodimers (Cao et al. 1997; Cainerca et al. 1999; Reymond et al. 2001).

There are different kinds of coiled-coils but the most common is a left-handed helix with a heptad repeat, where, due to the helical ~3.6 residues per turn, every seventh residue will point in the same direction. In a leucine zipper-type coiled-coil, this residue is a leucine, but other hydrophobic residues are also common. The repeated pattern promotes specific oligomerization, and a hydrophobic core will be formed, supported by electrostatic interactions between the polar residues facing outwards (Beck and Brodsky 1998; Mason and Arndt 2004).

The B30.2 domain

The C-terminal part of the Ro52 protein contains a predicted B30.2 domain (Henry et al. 1997; Henry et al. 1998) or SPRY (Ponting et al. 1997) domain. The domain was first identified in the ret finger protein (rfp) (Takahashi et al. 1988) and is therefore also called rfp-like domain. No general function has been mapped to the B30.2 domain, but in butyrophilin the B30.2 domain interacts with xanthine oxidase (Valivullah and Keenan 1989). No structure determination of a B30.2 domain has yet been published but a protein modelling study proposes that the domain consists of two immunoglobulin-like β-domains (Seto et al. 1999). The best fit for potential folds was obtained for an Ig-like fold, wherefore the suggested fold for the B30.2 domain is two Ig-domains.

The B30.2 domain has been identified in several different types of proteins except the TRIM protein, including butyrophilin and stonustoxin, a secreted toxin from Synanceja horrida. The B30.2 domain in Ro52 has not yet been associated with any
function except that a direct interaction between the IgG heavy chain and the Ro52 B30.2-like domain has been reported (Yang et al. 1999).

**Expression and cellular localization of Ro52**

Ro52 is a ubiquitously expressed protein with one alternative splice form, denoted Ro52β. In Ro52β the entire exon 4, comprising the most C-terminal part of the coiled-coil domain containing the leucine zipper structure (aa 169-245), is spliced off. Cellular Ro52β has so far only been detected at the mRNA-level and not at the level of synthesized protein (Chan et al. 1995; Buyon et al. 1997). It has been reported that the mRNA expression levels of the two splice forms differs between tissues in fetuses of certain age. The level of Ro52β was markedly increased in fetal hearts between week 14 and 16 of gestation (Buyon et al. 1997). The expression levels of the two splice forms have also been investigated in salivary glands of patients with Sjögren’s syndrome, but no significant difference could be seen between patients and healthy controls for either of the splice forms (Bolstad et al. 2003). The expression of Ro52 has also been reported to be elevated in the brain microvasculature (Shusta et al. 2003), but the relevance of this observation in context of Ro52 as a disease associated autoantigen still has to be defined.

The intracellular localization of Ro52 has been investigated in several reports with different methods. In 1993 the localization of Ro52 was investigated by fractionating cells into cytoplasmic and nuclear fractions and Ro52 was found mainly in the cytoplasm but to some extent also in the nucleus (Peek et al. 1993). The same result was obtained by using microinjection of protein into *Xenopus laevis* oocytes (Simons et al. 1994). A study with recombinant Ro52 fused to green fluorescent protein (GFP) (Pourmand et al. 1998), showed that full-length Ro52 is almost solemnly in the cytoplasm, but deletion constructs where the coiled-coil domain has been removed showed that both the C-terminal and the N-terminal end spreads evenly throughout the cell. These results indicates that the cytoplasmic retention of Ro52 is mediated by the coiled-coil region.

Ro52 has also been demonstrated to translocate in apoptotic or stressed cells in certain cell types. In keratinocytes Ro52 is translocated from the cytoplasm to the cell surface in response to UV-light and oxidative stress (LeFeber et al. 1984; Dörner et al. 1995b; Wang et al. 1999; Saegusa et al. 2002) or estradiol treatment (Furukawa et al. 1988). In apoptotic endothelial cells Ro52 is translocated to apoptotic blebs and associated with the plasma membrane (Ohlsson et al. 2002). This has also been demonstrated in apoptotic fetal cardiomyocytes (Miranda et al. 1998; Miranda-Carus et al. 2000).
Ro52 - Suggested functions and interaction partners

DNA-binding

Already when Ro52 was cloned and identified it was suggested to have a DNA or RNA binding potential based on the molecular composition with two zinc-fingers and a leucine zipper/coiled-coil domain (Chan et al. 1991; Itoh et al. 1991). When more knowledge was collected about different kinds of zinc-fingers, it turned out that the two zinc-finger motifs in Ro52 are not of the types that promote binding of nucleic acid, but instead usually promotes protein-protein-interactions (Borden 1998). A DNA-binding activity has, however, been suggested for Ro52 (Frank et al. 1995; Frank 1999) and even a consensus binding motif was described.

RNA-binding and association with Ro-RNP particles

Ro52 has been suggested to be associated with the Ro-RNP particle together with Ro60 and La (Ricchiuti et al. 1997), but in some studies it has been demonstrated that the Ro52 protein does not bind directly to RNA (Slobbe et al. 1992). Instead an association between Ro60 and Ro52 has been proposed (Slobbe et al. 1992; Pruijn et al. 1997). This protein-protein interaction has not yet been confirmed in other systems. Another mechanism for association of the Ro52 protein with the Ro-RNPs is via binding to the calreticulin protein (Cheng et al. 1996) which in turn would bind the hYRNA of the Ro-RNP particle. It is, however, not clear whether Ro52 is associated with the Ro-RNP particle or not.

Ro52 and ubiquitination

In line with many other RING-containing proteins (Trockenbacher et al. 2001; Linares et al. 2003), a role for Ro52 in ubiquitination has been suggested, and it has been shown that Ro52 can be ubiquitinated (Fukuda-Kamitani and Kamitani 2002) mainly by monoubiquitination but also by polyubiquitination. The ability to be covalently monoubiquitinated is a prerequisite for an E3 ligase and these data supports the suggestion of Ro52 as an E3 ligase, while polyubiquitination is usually a mark for degradation. Another supportive evidence for the role of Ro52 in ubiquitination is the described direct interaction between Ro52 and the de-ubiquitinating enzyme UnpEL (Di Donato et al. 2001).
Interaction between Ro52 and the heavy chain of IgG

A somewhat surprising protein-protein interaction between Ro52 and the IgG heavy chain has been reported (Yang et al. 1999; Yang et al. 2000). It is hypothesized that this interaction could be involved in the mechanism of antibody binding to apoptotic cells in for example fetal heart tissue in the case of congenital heart block. This protein-protein interaction is mapped to the C-terminal end of Ro52 containing the predicted B30.2 domain. The relevance of this interaction has yet not been confirmed in other studies.

Ro52 and homodimerization

The coiled-coil is one of the best studied and most common multimerization domains. In Ro52, the coiled-coil domain with the leucine zipper has been shown to promote a homodimer formation in vitro (Wang et al. 2001). In this study the dimerization of full-length Ro52 and the shorter splice version Ro52β was investigated and a dimerization was only observed with the full-length Ro52, but not with Ro52β lacking the leucine zipper. These results indicate that the leucine zipper is necessary for dimer formation.

Figure 8. Suggested interactions and functions of Ro52. Schematic view over Ro52 and proposed interactions. For interactions that have been mapped to a certain part of Ro52, these interaction sites have been visualized.
METHODOLOGICAL CONSIDERATIONS

A variety of methods have been used in this thesis and I will here give a short description of some of them, and also explain why we have chosen them.

**Limited proteolysis**
Structured parts of proteins are more resistant to proteolytic cleavage than unstructured parts. By performing a limited proteolytic cleavage of a protein and follow the obtained fragments over time, it is possible to identify the structured parts of the protein (Carey 2000). The fragments can be analyzed and identified by different methods, for example MALDI-MS or peptide sequencing by Edman degradation.

**Matrix Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI-MS)**
MALDI-MS is a mass spectrometric technique with little fragmentation of the analyzed molecules. It is very well suited for analysis of short peptides (Trauger et al. 2002) and we have used it mostly for detection of cleaved fragments after limited proteolysis. It has high sensitivity and requires only small sample quantities.

**Fluorescence spectrophotometry**
To determine the Zn$^{2+}$-binding affinity for the zinc-finger regions in Ro52, we used fluorescence spectroscopy (Nomura and Sugiura 2002; Uversky et al. 2002). We also used it for some folding studies. An advantage with fluorescence measurements is the high sensitivity and the low requirement for sample concentration.

**Phage display screening**
Phage display is a protein-protein interaction based method, which in our studies was used for antibody screening. Phages are made to express antibody fragment coded by cDNA from a library. The phages will express the antibody fragments on their outside and the phages are then screened against the antigen of interest, the phages that bind are selected, enriched and their cDNA cloned. This technique for obtaining recombinant patient derived antibodies is advantageous as it is difficult to generate patient derived monoclonal antibodies by traditional hybridoma technology.

**Circular dichroism spectrososcopy**
Peptides absorb left- and right-handed circular polarized light differently depending on their conformation. This is the basis for using circular dichroism spectroscopy for
analyzing secondary structure of peptides and proteins. We have used circular dichroism for folding analysis of our recombinant protein constructs and synthesized peptides. The circular dichroism measurements were made in the far ultraviolet region and gave information about the secondary structure composition of the analyzed peptide (Greenfield 2004). We also used circular dichroism for monitoring changes in peptide secondary structure upon addition of ligand, and for stability measurements by thermal unfolding.

**Analytical ultracentrifugation**
This method was used to determine the oligomeric state of recombinant protein constructs. The oligomeric state of the protein was analyzed by determining the sedimentation equilibria at different rotor speeds and sample concentrations. More information about ultracentrifugation methods can be found in (Lebowitz et al. 2002).

**Calcium level measurements with fluo-4**
The Ca$^{2+}$-dependent fluorophore fluo-4 was used for measurements of intracellular levels of free Ca$^{2+}$ in primary cultured cardiomyocytes. The cells were loaded with fluo-4 and the changes in Ca$^{2+}$ levels were monitored over time by using an inverted confocal microscope. This method was chosen for the possibility to follow the intracellular Ca$^{2+}$ fluctuations over time in living cells.

**Doppler echocardiography**
For detection of AV block in fetuses we used Doppler echocardiography (Andelfinger et al. 2001; DeGroff 2002), which measures the blood flow rate through the valves in the heart. The electrical impulses are difficult to measure on fetuses, but with Doppler echocardiography techniques the AV block can be visualized in utero.

**Mammalian two-hybrid system**
Two-hybrid system was used as in addition to analytical ultracentrifugation for analyzing homodimerization of Ro52. The mammalian two-hybrid system is based on activation of a reporter gene upon interaction between two proteins of interest fused to a DNA-binding domain and an activating domain, respectively. The advantage with this system for interaction studies is that it is performed in mammalian cells, which is close to the *in vivo* environment.

**Enzyme-linked immunosorbent assay (ELISA)**
ELISA was used frequently in the papers for antibody studies. The advantage with ELISA is that the investigated antigens can be natively folded and that the antibody
response in the tested sample can be quantified. ELISA was used both for detection of antibodies in sera and for characterization of monoclonal antibodies.

**Western blot**

Western blot was used for detection and characterization of antibodies, both from sera and monoclonal antibodies. Compared to ELISA the advantages with Western blot are the possibility to separate antigens on a gel before analysis, for example the separation of cell extracts. Disadvantages are the inability to use natively folded proteins and the lack of quantitative results.

**Immunofluorescence**

For visualization of antigens in cells, we used immunofluorescence staining. This is a good method for determining if the antigen is membrane associated or intracellular, and also for determining the intracellular localization.
AIMS OF THE THESIS

The Ro52 protein is one of the major autoantigenic targets in Sjögren’s syndrome. This thesis was focused on characterization of the Ro52 protein and antibodies directed against this antigen. Congenital heart block in children of mothers with anti-Ro52 antibodies is a disorder where the anti-Ro52 antibodies have been suggested to have a pathological role, and to investigate this role further was also a major part of the thesis work. The more specific aims addressed in this thesis work were the following:

- To investigate if any structurally stable domains could be found in Ro52, and if such domains correspond to the predicted domains based on sequence similarity and bioinformatic analysis.
- To characterize the experimentally defined domains and determine if they appeared to be functional in Ro52.
- To analyze how the immunodominant parts of Ro52 correspond to the found domains.
- To identify and clone human recombinant antibodies against the Ro52 protein with similar specificity as the antibodies associated with development of congenital heart block.
- To characterize the cloned anti-Ro52 antibodies and to identify and map congenital heart block associated epitopes.
- To investigate the mechanism for induction of congenital heart block by developing a suitable animal model and study the effect of specific anti-Ro52 antibodies on cardiomyocytes.
RESULTS AND DISCUSSION

Molecular structure of the Ro52 protein

There have been many studies on the domain structure and the relevance of different domains for suggested functions of Ro52. However, all the studies have been based on predicted domains and domain borders identified by sequence similarity between Ro52 and other proteins. It has never been empirically analyzed if the predicted domains really are structured, stable and present in the native protein. To determine which stable parts could be found in Ro52 we used bacterially expressed fusion-tagged Ro52 in limited proteolysis experiments. We found two stable entities which covered aa 7 to 121 and aa 122 to approximately 260 (Figure 9). Compared to the predicted domains, the N-terminal fragment covers both the zinc-fingers and the aa 122-260 fragment spans the complete coiled-coil region. This first analysis of stable regions in Ro52 (Paper I) was performed with Coomassie stained SDS-PAGE as detection system, which makes it difficult to follow more extensive degradation of the fragments. For a more detailed investigation of the further degradation of the fragments, we used MALDI-MS as detection system in paper II.

Figure 9. Proteolytic degradation of recombinant Ro52 by trypsin cleavage. Trypsin cleavage of recombinant Ro52 generates two fragments comprising the zinc-finger region and the coiled-coil domain. The fragments are further cleaved upon more extensive trypsin cleavage.
From this first study it could be concluded that the two zinc-fingers formed one stable entity and the coiled-coil domain another one. The C-terminal part of the Ro52 protein was problematic to maintain in solution with its native fold. Already during expression in *E. coli* the C-terminal part was degraded and the full-length protein was hardly detectable on SDS-PAGE. A subcloned construct with the C-terminal part of Ro52 alone could not be refolded to its native state without extensive precipitations.

**Characterization of the \( \text{Zn}^{2+} \)-binding motifs in Ro52**

It has been shown that both of the putative zinc-fingers of Ro52 have \( \text{Zn}^{2+} \)-binding affinity (Pourmand and Pettersson 1998), but in our study (Paper II) we wanted to determine the affinity for the different \( \text{Zn}^{2+} \)-binding sites and also investigate the structure of the zinc-fingers as well as the \( \text{Zn}^{2+} \)-dependence of the structure. \( \text{Zn}^{2+} \)-binding was investigated by three different methods: circular dichroism, limited proteolysis followed by MALDI-MS analysis and fluorescence spectrophotometry. The \( \text{Zn}^{2+} \)-binding affinity for the two predicted zinc-fingers were investigated by synthesizing three peptides based on bioinformatic comparison of different TRIM proteins using the consensus sequence. The three peptides covered the RING-finger, the region between the two zinc-fingers denoted the middle-region and the B-box, respectively. The \( \text{Zn}^{2+} \)-binding constants were determined by fluorescence spectroscopy, which was a favorable method because of the presence of one strongly fluorescent tryptophane in each of the \( \text{Zn}^{2+} \)-binding peptides. As expected, two \( \text{Zn}^{2+} \)-binding sites could be detected in the RING-finger peptide, one site with one magnitude higher \( \text{Zn}^{2+} \) affinity than the other (\( k_{d1}=1.6 \times 10^{-9} \text{ M} \) and \( k_{d2}=6.3 \times 10^{-8} \text{ M} \)). Only one \( \text{Zn}^{2+} \)-binding site could be seen in the B-box peptide and it had a much lower affinity (\( k_{d}=3.5 \times 10^{-6} \text{ M} \)) than the ones in the RING peptide.

The \( \text{Zn}^{2+} \)-binding was also determined for a HIS-tagged construct spanning the whole \( \text{Zn}^{2+} \)-binding region (aa 1-128) and then the best fit was for three or four \( \text{Zn}^{2+} \)-binding sites. The total \( \text{Zn}^{2+} \)-binding affinity was higher than the sum for the individual peptides. The extra binding site could be explained by unspecific binding to the HIS-tag and the higher total \( \text{Zn}^{2+} \) affinity could to some extent be explained by \( \text{Zn}^{2+} \)-binding to the HIS-tag. However, that contribution cannot explain the whole increase in affinity. Instead, it seems like the folding of the two \( \text{Zn}^{2+} \)-binding motifs when they are expressed together enhances the binding of \( \text{Zn}^{2+} \). This difference in folding is also shown by the burial of the tryptophanes in response to \( \text{Zn}^{2+} \)-binding. The single \( \text{Zn}^{2+} \)-binding site in the B-box is consistent with other reports (Borden et al. 1995b), and the sequential binding of
Zn$^{2+}$ to the RING-finger also correlates with investigations of other RING-fingers (Roehm and Berg 1997).

A circular dichroism study of both the peptides and the whole region was performed with and without addition of Zn$^{2+}$. This was somewhat problematic because of the risk for precipitation of zinc hydroxide at high pH versus the risk for protonation of histidines at low pH. Another factor was the stability of the zinc-finger construct that was dramatically lower at low pH and also a tendency for precipitations at low pH. As a result of these problems, only the individual peptides could be analyzed by far UV circular dichroism and use of reducing agents was also inhibited by the interference they caused in the low wavelength area. Despite the difficulties, a clear effect of Zn$^{2+}$ addition could be seen, both for the RING-finger peptide and the B-box peptide. The RING-finger displays an increase in $\alpha$-helix content at addition of Zn$^{2+}$ and consists mainly of $\alpha$-helix and random coil, while the B-box displays a mixture between $\beta$-sheet and random coil. Surprisingly, the middle-region, which has a high propensity for $\alpha$-helical fold, does not appear helical by circular dichroism. This could have several explanations, but the most likely reason is that it requires the flanking Zn$^{2+}$-binding regions or other interacting protein domains to adopt a correct fold.

Analysis of the coiled-coil-domain

After identifying the coiled-coil stretch as one stable entity it was subcloned (aa 121-258) into a 6xHIS-tag vector and expressed in E. coli. To obtain large quantities of protein, the expression was performed under denaturing conditions and the protein was refolded to its assumed native state. Secondary structure analysis by circular dichroism confirmed the predicted predominant $\alpha$-helical fold of the coiled-coil domain. Thermal unfolding of the coiled-coil-domain showed a cooperative unfolding of the protein fragment upon heating, which is characteristic for a coiled-coil structure (Naik et al. 2001). The $\alpha$-helical fold of the coiled-coil domain also supports the predicted leucine zipper, which is dependent on an $\alpha$-helical fold of the peptide (Kass et al. 2003).

The coiled-coil domain was also expressed together with the N-terminal part of Ro52 (altogether spanning aa 1-258) to give information about possible inter domain interactions. However, no such interaction could be seen in circular dichroism analysis. Limited proteolytic cleavage of the zinc-finger-coiled-coil construct was also performed and to detect smaller fragments from a more extensive cleavage, MALDI-MS was used as detection system. By analyzing the cleaved fragments with the higher sensitivity that the MALDI-MS provides it was possible to follow the sequential cleavage of the coiled-
coil stretch. The same cleavage of the coiled-coil domain from the zinc-finger stretch could be seen again, but also a further degradation that separates the N-terminal part of the coiled-coil (aa 121-177) from the C-terminal part (aa 178-253) (Figure 9), which indicates a less folded stretch of amino acids within the coiled-coil domain. This corresponds very well to the secondary fold predictions which show a low propensity for coiled-coil at aa 167-180 (Figure 10A), and that could make cleavage sites present in this stretch exposed and more accessible for proteolytic cleavage (Figure 10B).

**Figure 10. Coiled-coil prediction of Ro52 and hypothetical folding of the coiled-coil domain.**
The upper panel shows a prediction of coiled-coil probability for the Ro52 protein. The lower panel shows a hypothetical two part coiled-coil domain with amino acid 167-180 exposed. The approximate p200-peptide stretch of the coiled-coil is dark grey. The coiled-coil prediction was done using the COILS-software (Lupas et al. 1991).
Protein-protein interaction studies and clues to function

Intramolecular interactions

By the identification of a functionally folded coiled-coil domain, a RING-finger with secondary fold and Zn$^{2+}$-binding affinity corresponding to other RING-fingers as well as B-box with a secondary fold that correlates with the determined B-box structure (Borden et al. 1995b) and a binding affinity for Zn$^{2+}$, Ro52 can be put in the TRIM protein family, not only based on sequence homology but also on experimental results. The coiled-coil domain is well known to promote protein-protein interactions in both homo- and hetero-multimerization systems (Jensen et al. 2001; Paris et al. 2003) and in Paper I we investigated the potential homodimer formation for the coiled-coil domain by analytical ultracentrifugation and for the full length protein by mammalian two-hybrid system.

Ultracentrifugation analysis showed that the coiled-coil region expressed by itself exist in solution in a monomer-dimer equilibrium. The homodimerization appeared to be fairly weak with a dissociation constant of approximately 25 µM. The same conclusion can be drawn from the mammalian two-hybrid system experiments, which also displayed a weak dimerization between two Ro52 molecules. That a stronger dimerization was not observed could be an indication of a potential protein-protein interaction with another protein, most probably via a corresponding coiled-coil domain.

Dimerization of Ro52 via the coiled-coil domain has been reported before from in vitro experiment (Wang et al. 2001) and a yeast two-hybrid system (Reymond et al. 2001) with full length Ro52. Together this could indicate that the coiled-coil domain is necessary for dimerization but that the interaction is enhanced by the presence of either the N-terminal or the C-terminal flanking regions. The weak homodimerization can also be an indication of a potential heterodimerization, because the weak association shows that the coiled-coil domain is functional but maybe Ro52 is not the optional interaction partner. Interactions with other proteins have been reported, for example interaction with the de-ubiquitinating enzyme UnpEL (Di Donato et al. 2001). The interaction with UnpEL was showed to be dependent on the presence of the leucine zipper stretch.

Clues to function

According to our results, the RING-finger, B-box and the coiled-coil domain are all stable, structured and functional domains in Ro52. This confirms that the protein really belongs to the TRIM family. So far the TRIM proteins have not been linked to a single
function but several members of the protein family are involved in transcription regulation (Tissot and Mechti 1995; Shimono et al. 2000). A common feature for many members is also their ability to function as proto-oncogenes in different tumor forms (Grignani et al. 1994; Le Douarin et al. 1995). Among the increasing numbers of proteins involved in the ubiquitin dependent degradation of proteins, the RING-finger is a very common motif, and for a group of E3 ubiquitin ligases it has been proven to be required for ubiquitination of the target protein (Freemont 2000). Among those RING-dependent E3 ligases there are members of the TRIM family (Horn et al. 2004), and Ro52 can be mono ubiquitinated (Fukuda-Kamitani and Kamitani 2002) which is a characteristic feature for E3 ligases.

There is a report from 2003 about the involvement of Ro52 in the CD28 mediated IL-2 production in T cells (Ishii et al. 2003), and that over-expression of Ro52 would increase the level of IL-2 production in response to α-CD28 stimulation. That Ro52 would act as a direct transcription regulator is perhaps not that probable, because of the lack of known DNA-binding motifs. The zinc-fingers present in the Ro52 protein are known to be protein interaction motifs and not DNA-binding motifs. This is also supported by our data from limited proteolysis experiments with Ro52 in presence of DNA. When a DNA-binding protein binds to its target DNA, a general stabilization of the interacting protein domains can be seen (Pursglove et al. 2004). In our experiments we have used both the Ro52 binding DNA consensus sequence derived from an earlier study (Frank 1999) as well as unspecific DNA in high concentration. No stabilization of the zinc-finger stretch from Ro52 could be seen with either the specific or unspecific DNA by limited proteolysis experiments. However, Ro52 could very well act as an indirect regulator of transcription, for example by participating in a more complex multimeric protein like the KAP-1 association to KRAB (Peng et al. 2000), or by functioning as an E3 ubiquitin ligase and tag a transcription repressor factor for degradation by ubiquitination.

Ro52 and antibody-antigen interaction

Immunodominant epitopes in Ro52 co-localize with stable structural domains

The epitopes recognized by antibodies have been mapped in different studies before with different methods (Bozic et al. 1993; Blange et al. 1994; Buyon et al. 1994; Frank et al. 1994; McCauliffe et al. 1994; Ricchiuti et al. 1994; Kato et al. 1995; Dörner et al. 1996; Pourmand and Pettersson 1998). In almost all studies the immunodominant epitopes are
found within the coiled-coil region of Ro52 (Wahren-Herlenius et al. 1999), and in some studies epitopes were also found in the N-terminal part of the protein. In paper I we confirm that most of the tested SS patient sera have reactivity against the coiled-coil domain and around half of the patient sera react against the zinc-finger construct (aa 1-128), but only with reducing conditions as reported before (Pourmand and Pettersson 1998). None of the patient sera recognized the isolated B30.2-construct (aa 261-275), which corresponds well with most other reports. However, the B30.2-construct was not possible to refold in our system and therefore conformation dependent epitopes would not be recognized. An incorrect fold of certain stretches could also disrupt linear epitopes. The conclusion that can be drawn from our results is that the immunodominant epitopes co-localize with the structurally stable domains in Ro52.

**Antibodies directed against the zinc-finger region of Ro52**

No autoantibodies against the zinc-finger region of Ro52 could be found in sera from SS patients when analyzed by normal immunoblot or ELISA, but if the antigen was reduced prior to incubation with sera, 60-70% of the patient sera showed reactivity against the zinc-finger region (paper I). The results correlate well with earlier studies using different Ro52-constructs (Pourmand and Pettersson 1998). The recognition of the zinc-finger region was not Zn$^{2+}$-dependent and addition of Zn$^{2+}$ without reducing condition did not affect the antibody binding. The inability for the antibodies to bind the non-reduced antigen can be due to blocking of the binding sites by the formation of sulfur bridges between the numerous cysteine residues present in the zinc-finger region of Ro52.

In paper II the three peptides representing the RING, B-box and middle regions were also analyzed by ELISA with sera from SS patients. In this study patients positive for the whole zinc-finger construct were chosen. None of the three peptides were recognized by any of the patient sera, not even with reducing conditions, which was quite surprising. One explanation can be that the recognized epitopes are overlapping the peptide borders or that an epitope is located in the very N-terminal of Ro52, partly outside the RING consensus sequence, which is supported by another study (Ricchiuti et al. 1994). Nine rat-anti-human Ro52 monoclonal antibodies specific for the zinc-finger region were also tested against the three peptides in paper II, and all nine were specific for the middle-region between the RING-finger and the B-box. The monoclonal antibodies were supernatants from hybridomas based on B cells from Ro52-immunized mice. If one is to speculate about the difference in reactivity between the monoclonal antibodies and the patient sera the most plausible explanation is probably set up for immunization of the rats and the screening of the antibody producing hybridomas. If the
antigen used for immunization does not have the right native conformation, the obtained monoclonal antibodies will not be able to recognize conformational epitopes. The same is also valid for the screening; if the hybridomas are screened against a wrongly folded antigen, the clones recognizing a conformational epitope will be screened out. The middle region between the zinc-fingers in Ro52 has a low secondary structure prediction and no Zn$^{2+}$ or reducing agent dependency folding and will therefore be more likely not to contain a complete conformational epitope.

**Anti-coiled-coil autoantibodies**

In a study concerning autoantibodies associated with CHB, mothers of fetuses affected by the condition displayed often high titers of antibodies directed against a peptide spanning aa 200-239 of Ro52 (denoted p200) (Salomonsson et al. 2002). This peptide represents a part of the coiled-coil domain and contains the complete leucine zipper. To investigate the relevance of this finding, monoclonal antibodies were cloned by screening scFV combinatorial antibody phage display libraries based on RNA obtained from bone marrow and peripheral blood lymphocytes from autoimmune patients (Hoet et al. 1998; Zampieri et al. 2003) (paper III). The antibody clones were screened and selected for their recognition of both Ro52 and p200. Two different antibody clones specific for p200 were obtained, S3A8 and M4H1, and they were transferred to an *E. coli* expression system. Both recombinant antibody clones bound to Ro52 and p200 in immunoblot and ELISA, and they bound endogenous Ro52 in cell extracts from human HeLa cells and murine 3T3. They also stained both 3T3 and HeLa cells in immunofluorescence.

**Mapping of the S3A8 and M4H1 antibody epitopes by alanine scanning**

Already in the initial characterization of the recombinant anti-p200 antibodies in paper III, we found that the antibodies bound well to p200 but did not bind to the highly overlapping peptide p197 (spanning aa 197-232). To investigate if the reason was that the epitope was located in the seven non-overlapping C-terminal amino acids in p200 we performed an alanine scan of this region (paper IV). Seven peptides derived from p200 was synthesized with one of the seven most C-terminal aa substituted for an alanine in each peptide. The peptides were used in ELISA and the alanine substitution of aa 234-239 did not alter the antibody reactivity while substitution of the aspartic acid at position 233 totally abolished the antibody binding. This indicates that aa 233 is the most C-terminal anchor amino acid in the M4H1 epitope, and if the epitope stretches further there are no residues crucial for antibody binding (Figure 11). For M4H1 this explains the
difference in recognition of p200 and p197, because the D233 is missing in p197. For the S3A8 epitope the alanine scan did not give a clear answer, instead some substitutions increased the antibody binding instead of decreasing it. Alanine substitution of D233 reduces the antibody binding with about 50%, indicating that it is not a crucial amino acid as it is for the M4H1 binding.

![S3A8-epitope and M4H1-epitope](image)

**Figure 11. The suggested localization of the S3A8 and M4H1 epitopes within the p200 peptide stretch.** According to the fine mapping of the S3A8 and M4H1 antibody epitopes, the S3A8 antibody seems to recognize a conformational epitope covering the middle of the p200 peptide. The M4H1 epitope appears to be a linear epitope in the very C-terminal end of p200.

**Mapping of the S3A8 epitope by structure altered peptides**

When the alanine scan displayed that the S3A8 epitope is not a linear epitope we assumed that it was a conformational epitope. When modeling the p200 stretch with a known leucine zipper as template we noticed that there were several amino acids interfering with the leucine zipper formation, depending on their charge and hydrophilicity/hydrophobicity. Notable was also that the part of a hypothetical leucine zipper pointing outward from the hydrophobic core contained a stretch of negatively charged glutamic acids.

To define the S3A8 epitope we made a synthetic peptide where three charged amino acids in the leucine zipper core were substituted for hydrophobic ones to make a more favorable leucine zipper (pZIP). We also synthesized a peptide where the stretch of glutamic acids on the outside of the leucine zipper was substituted for basic or non-charged amino acids (pOUT). Circular dichroism analysis of these peptides showed that the pZIP peptide has a much higher alpha-helical content than p200 and it is more stable in denaturing experiments. The pOUT peptide lost most of its alpha-helical content and...
the secondary structure consists mainly of random coil in solution. In ELISA, M4H1 antibodies bound as well to the pOUT peptide as to p200, which confirms the conclusion that the M4H1 epitope is not structure dependent (paper IV). The binding of M4H1 to the pZIP peptide was decreased to about 25% of the binding to p200 and this reduction can be due to a masking of the epitope because of the tighter dimer formation of two pZIP peptides forming a leucine zipper.

The S3A8 antibodies bound neither the pZIP or pOUT peptide. This could be explained by the point mutations of amino acids in the peptides but then S3A8 should bind p197 as good as p200 which is not the case. Instead it indicates that the S3A8 epitope is dependent on the secondary structure of the peptide. The secondary structure of the p200 peptide consists of alpha-helix and to a large part random coil, but both when the contribution of alpha-helix is decreased (pOUT) or increased (pZIP) the antibody binding is disrupted.

The results from ELISA analysis of the different peptides indicated, as already discussed, that the M4H1 epitope is a linear epitope with D233 as the most C-terminal crucial amino acid while the S3A8 epitope appears to be a structure dependent epitope spanning the middle of p200 (Figure 11). The binding of the peptides to the ELISA plate could be thought to interfere with the peptides conformation and that it does not give correct information. Therefore we also performed a type of antibody protection assay based on limited proteolysis and detection by MALDI-MS. In these experiments we showed that the p200 peptide is protected from V8 proteolytic cleavage by addition of S3A8 or M4H1 antibodies. The difference in binding of the two antibodies was also clearly visible in these experiments. The middle stretch of p200 (aa 208-228) was much better protected by S3A8 than by M4H1, while the C-terminal part (cleavage sites E28, E32, D34) was better protected by M4H1 than by S3A8.

**Anti-p200 antibodies from mothers of CHB-affected children mainly show S3A8 specificity**

After determining that the two monoclonal antibodies from paper III recognized two different epitopes within the p200 peptide it was of main importance to investigate what kind of antibodies are most prevalent in mothers of children affected by CHB. In paper IV we tested sera from 12 mothers of CHB-affected children by ELISA with the mutated peptides as well as p200 and p197. The recognition patterns for the sera were as expected more complex than for the monoclonal antibodies This is dependent on the presence of multiple specificities in the sera. The patient sera often had some reactivity against the pZIP peptide which differs from the S3A8 recognition pattern, but otherwise the pattern corresponds better to the S3A8 pattern than the M4H1. Especially the lack of binding to
the pOUT peptide is interesting, because the M4H1 antibodies recognize it to the same extent as the p200-peptide itself. The same recognition pattern could also be seen in sera from the children affected by CHB (paper V). These findings indicate that the disease-associated antibodies in CHB are not only anti-p200 specific but also specific for a conformational epitope recognized by S3A8. The importance of these antibodies will be discussed in more detail later.

Anti-p200 antibodies and congenital heart block

Anti-p200 autoantibody levels correlate with development of CHB

The blockage of the signal transduction system in the fetal heart can be analyzed by measuring the AV conduction times. Based on the study showing that mothers of children with CHB has high levels of anti-p200 antibodies (Salomonsson et al. 2002) we investigated if the level of anti-p200 antibodies correlates with the development of CHB in the fetus, displayed as a prolongation of the AV time. The AV times were measured using two different Doppler echocardiography techniques (Sonesson et al. 2004). A significant correlation between high maternal anti-p200 levels and prolongation of the AV conduction times was detected, with mothers of fetuses developing AV blocks among the ones with highest anti-p200 antibody levels. Even stronger correlation was seen between the prolonged AV time and a quote for level of antibodies against p200 and p176 (p200/p176). Anti-p176 is the antibody specificity that was found dominating in mothers with unaffected fetuses (Salomonsson et al. 2002). These results confirm the connection between the presence of anti-p200 antibodies and the development of heart block in the fetal heart, and they also indicate a direct dependence on the level of autoantibodies.

The association of anti-Ro52 autoantibodies and CHB has been investigated and reported (Buyon et al. 1993; Julkunen et al. 1993; Brucato et al. 1995; Dörner et al. 1995a), but the significance of antibodies directed against one specific epitope has not been stated before. In several studies sera from Ro/La positive mothers have been analyzed against native or recombinant full-length Ro52. In that experimental set up it is not possible to distinguish between antibody reactivity against for example p176 (high prevalence in mothers of healthy children) and p200 (high prevalence in mothers of children with CHB) (paper V; (Salomonsson et al. 2002). In a study where shorter fragments of the Ro52 protein were analyzed (Dörner et al. 1996), the strongest immunogenic part of the protein was found at the N-terminal, aa 1-245, and especially the stretch between aa 197 and 245. However, this dominant antigenicity was not
especially associated with CHB in fetuses, but more a common feature in all patients with anti-Ro52 antibodies.

Considering that it seems to be a certain epitope within the p200 stretch (the epitope recognized by S3A8-like antibodies) that is associated with CHB, it is not surprising that the association has not been detected in other studies. Studies using large Ro52-fragments would not be able to distinguish the S3A8-like antibodies from antibodies present in both mothers of healthy children and mothers of children with CHB. If short overlapping peptides would be used instead, the disease associated epitope could easily be missed out because of the peptides would not be long enough to cover a conformational epitope or would be too short to obtain the right fold, as we have shown in paper III and IV using the p197 and p200 peptides.

**Peptide p200 immunized rats as an animal model for CHB**

There are several established animal models for CHB (Boutjdir et al. 1997; Miranda-Carus et al. 1998; Xiao et al. 2001a), but to investigate if our findings of p200 autoantibodies as the disease mediating antibodies were reproducible in an *in vivo* system we developed a rat model. Female DA rats were immunized and boosted with synthesized p200 until stable levels of anti-p200 antibodies were reached, they were then mated and the AV conduction time in the newborn pups were measured by electrocardiogram. About 20% of the immunized pups developed a first degree heart block while none of the pups from control immunized rats did. The fact that none of the pups from p200-immunized rats developed a higher degree block can have several explanations. First of all, immunization with free peptides is far from the optimal way of immunization. It is also crucial that the antibodies produced in response to the immunization are of the right specificity (S3A8-like specificity according to results in paper IV). The specificities obtained in the immunized rats vary, which can be seen by the binding reactivities against the mutated peptides used in paper IV. The genetic background of the DA rat can also be a reason for the outcome of the heart block. Despite the absence of high degree heart blocks in the pups, the model shows that the anti-p200 antibodies can induce the development of an AV block in the pups.

In a mouse model based on immunization with recombinant full-length Ro52 (Miranda-Carus et al. 1998), the incidence of first degree AV block was lower than in our p200-immunized DA rat model, but higher degree heart blocks were detectable in 2 of 56 pups. The differences in incidence and severity of induced heart block in pups may be explained by the different species used, but it can also be explained by differences in administration of the immunization. The induction of AV block II and III in the mouse
model but not in our rat model can be due to the fact that immunization with a recombinant protein gives a better immune response than immunization with shorter peptides, and therefore often higher antibody levels. Another study using the same BALB/c mouse model displayed a much higher incidence of CHB in the pups (Boutjdir et al. 1997), maybe due to differences in immunization and preparation of the immunization antigen. Studies in rabbit pups of mothers immunized with human recombinant Ro52 displayed a CHB-incidence below 10%, while approximately 20% of the pups were still-born (Xiao et al. 2001b). The cause of death for the stillborn pups was not determined but some deaths might have been caused by complete AV blocks. The incidence of CHB corresponds well with the results from our studies in rats.

The results from the studies in different animal models also give information about the cross-reactivity of anti-Ro52 antibodies between different species. In all the mentioned animal models, human recombinant Ro52 has been used for immunization and antibodies directed against the human antigen has been determined to be associated with the induction of CHB in rat, mouse and rabbit, as well as in humans. This indicates that the autoantibodies needed for development of CHB most probably is directed against a highly conserved part of the Ro52 protein. These speculations fit well with the suggestion of anti-p200 antibodies as the pathogenic antibodies, as the leucine zipper is one of the most conserved region parts of Ro52 between species. If the CHB is initialized by anti-Ro52 antibodies cross-reacting with a surface antigen on cells in the heart, the ability of anti-human Ro52 antibodies to cause CHB in different species also indicates that the recognized epitope on the surface antigen must be well conserved between species.

**Anti-p200 antibodies have a direct pathogenic effect on cardiomyocytes in vitro**

After identifying the importance of certain anti-p200 antibodies for the development of CHB both in patients and in an animal model, one important question was how it can cause the heart block. Since the heart is the target organ for CHB we established *in vitro* cultures of primary myocytes from newborn rats. First we wanted to investigate whether the recombinant anti-p200 monoclonal antibodies S3A8 and M4H1 could bind to the surface of the myocytes as this would be a prerequisite for any direct pathogenic effects. In immunofluorescence experiments S3A8 antibodies bound to the myocyte cell surface, while M4H1 did not bind.

Cardiomyocytes in cultures contracts spontaneously due to their endogenous pacemaking ability. These contractions are regulated by the internal Ca$^{2+}$ levels and Ca$^{2+}$ is also an important regulator of induced contractility. To investigate the effects of
antibodies binding to the cardiomyocytes we monitored the intracellular calcium level by loading the cells with the fluorescent calcium indicator fluo-4 and follow the cells over time with confocal microscopy. Upon addition of S3A8 antibodies an initial increase in the pace of Ca^{2+} oscillation was noted, followed by a decrease in Ca^{2+} oscillation frequency and an accumulation of intracellular Ca^{2+}. After one hour, all cells had stopped Ca^{2+} oscillation and died. Cells treated with M4H1 or PBS only did not show this effect, but were instead still oscillating and contracting at spontaneous pace even after 24 hours of treatment.

Interestingly, sera from p200-immunized rats also had an effect on the primary cultured cardiomyocytes, comparable to the effect of S3A8. However, some cells were still contracting and showed Ca^{2+} oscillation after 24 hours, indicating that the effect of the rat sera was not as profound as the S3A8 antibodies. This can of course be a matter of concentration, though the characterization of the rat sera revealed, as mentioned above, that the sera contains many different antibody specificities and not only the S3A8-like antibodies.

The connection between autoantibodies and dysregulation of the Ca^{2+} homeostasis has been investigated before and it has been shown that sera from mothers of children affected by CHB can inhibit the function of L-type calcium channels in rats (Boutjdir et al. 1998) and in a model with calcium channels expressed on transfected cells (Xiao et al. 2001a). The expression of L-type calcium channels is also down-regulated by antibodies in sera from mothers of children with CHB (Boutjdir et al. 1997; Boutjdir et al. 1998; Qu et al. 2001). From our results we can not yet draw any conclusions about the molecular mechanism behind the disturbance of the Ca^{2+}-homeostasis in the cardiomyocytes in response to binding of S3A8 antibodies; if there is a direct binding to a calcium-related channel or if they interfere with the regulatory system.

Ca^{2+} overload is a well known trigger of apoptosis in cells. TUNEL staining and staining for caspase-3 confirmed that the intracellular accumulation of Ca^{2+} in the S3A8 treated cardiomyocytes induced apoptosis in the cells and that they die by this pathway. Other studies has shown that Ro52 molecules translocate to the cell surface in apoptotic fetal cardiomyocytes, where they become accessible to autoantibodies (Miranda et al. 1998; Miranda-Carus et al. 2000), and this translocation of Ro52 has also been displayed in apoptotic epithelial cells (Ohlsson et al. 2002). This translocation has been suggested as the induction of the forming heart block, but based on our studies with S3A8 antibodies the starting point is most probably binding of autoantibodies to a cross-reactive surface antigen on the cardiomyocytes. The binding of the antibodies will affect the Ca^{2+}-homeostasis which will in turn induce apoptosis in the affected cells. If the Ro52 molecules translocated to the cell surface, as shown in other studies, it will lead to a more profound antibody accumulation that could be the driving force for the forming
inflammation site. Translocation of Ro60 and La to the cell surface of apoptotic cells has also been shown, and the binding of antibodies directed against these antigens could also have a role in the continuing inflammatory response (Miranda-Carus et al. 2000; Ohlsson et al. 2002; Tran et al. 2002b). The inflammatory response and the following induction of fibrosis could then be causing the actual AV block (Buyon and Clancy 2003; Clancy and Buyon 2004; Clancy et al. 2004).
CONCLUSIONS

The focus of this thesis has been the characterization of Ro52, one of the major autoantigens targeted in the autoimmune disorder Sjögren’s syndrome. Analysis of the autoantibodies directed against the Ro52 protein mainly in patients with Sjögren’s syndrome and mothers of children suffering from congenital heart block has also been performed. We have been working with a variety of methods spanning from the areas of biochemistry and biophysics to immunologic methods like ELISA, cell culturing and animal models. This has given us the opportunity to obtain knowledge about Ro52 ranging from molecular characterization to the importance of anti-Ro52 antibodies in patients.

The molecular characterization of Ro52 has made it possible to state that the RING-finger, the B-box and the coiled-coil domain, predicted in Ro52 by sequence homologies, are present in the Ro52 protein as stable and functional domains. The investigation of the zinc-fingers confirms the binding of two Zn$^{2+}$ ions to the RING-finger and one Zn$^{2+}$ ion to the B-box. The Zn$^{2+}$ affinity and the secondary structure of the predicted zinc-fingers also correspond to reports concerning the same domains in other proteins. The coiled-coil was also identified as a functional domain with the secondary structure predicted by bioinformatics analysis. The coiled-coil domain was determined to promote formation of homodimers, however, the association constant indicates that it is a fairly weak dimer. This can be an indication of a preferred association to an as yet unknown interaction partner, or a dependence on dimer/monomer equilibrium for the function of the Ro52 protein.

Immunological characterization of the Ro52 protein and its stable domains show that the immunodominant parts of Ro52 correspond to the structurally stable domains, and confirms the presence of conformational epitopes in the N-terminal part spanning the two zinc-fingers and an immunodominant stretch covering the leucine zipper.

We have also cloned and characterized recombinant antibodies directed against the p200 stretch of Ro52 (aa 200-239) that has been shown earlier to be involved in the development of CHB in fetuses of mothers who are positive for these antibodies. We have elucidated that there is a certain antibody specificity that seem to be highly associated with the induction of CHB. Our results show that the recombinant antibody S3A8 antibody can bind to the cell surface of cardiomyocytes in primary cultures and that the binding causes a dysregulation of the Ca$^{2+}$-homeostasis, which is followed by apoptosis. Anti-Ro52 antibodies from mothers of children with CHB mainly display similar reactivity pattern as the S3A8 antibodies when tested against a set of epitope defining mutated peptides. We were also able to map the disease associated S3A8 epitope
to a certain stretch of the p200 peptide, and it seems to be a conformational epitope dependent on the rigidity/flexibility of the alpha helical fold of the peptide.

Anti-p200 antibodies induced by immunization with the p200 peptide in female DA rats induce CHB in their pups, indicating a pathogenic role of these antibodies in the development of CHB. The binding of anti-p200 antibodies to the cardiomyocytes followed by induction of apoptosis might trigger an active inflammatory response in the heart, resulting in fetal conduction abnormalities detected as AV block. This pathway suggests the presence of a cross-reactive antigen on the cell surface of the cardiomyocytes, and an important step to elucidate the whole mechanism behind the heart block is to identify the target molecule. The characterization of the S3A8 antibody epitope gives some hints about the target, but the dependence on conformation instead of primary structure complicates the theoretical screening process.

The main focus of a continuation of the studies in this thesis would be to determine the cellular function of the Ro52 protein and to identify potential interaction partners. In the area of congenital heart block and anti-Ro52 antibodies, the main focus would of course be, as mentioned above, to identify the cell surface molecule that cross-reacts with anti-p200 antibodies binding to the cardiomyocytes.
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