A radical view on the development of myositis

Sander van Dooren

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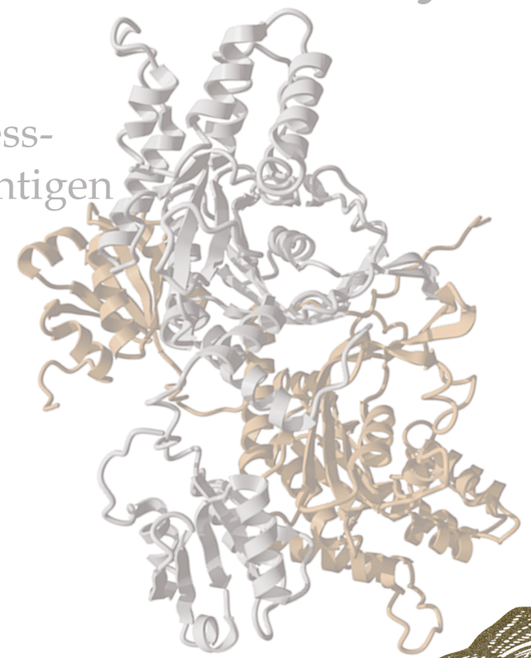
Oxidative and nitrosative stress-induced autoantigen modifcations



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A radical view on the development of myositis

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Oxidative and nitrosative stress-induced autoantigen modifications

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Chapter 1

General introduction

An increasing number of individuals are suffering from autoimmune diseases, especially in Western countries. Since most disorders eventually affect everyday tasks, it is of great interest to elucidate the etiology and develop adequate therapy for the different autoimmune diseases. Intensive research in the last decades revealed that both genetic and environmental factors are involved in the development of autoimmunity. Most autoimmune diseases are characterized by the presence of autoantibodies directed against a variety of 'self'-molecules, so-called autoantigens, in patients' sera. Some of the autoantibodies, which frequently are produced already early in the disease, are specifically associated with a particular autoimmune disease and therefore can be applied as a diagnostic biomarker. Also for myositis, a rare autoimmune disease in which skeletal muscle tissue is chronically inflamed, a number of biomarkers that are helpful in diagnosis and prognosis have been described. The first two chapters of this thesis summarize our current knowledge on the processes that are associated with the development of myositis and provide an overview of autoantibody-autoantigen systems associated with this disease and autoantibody detection techniques.

Myositis / Idiopathic inflammatory myopathies (IIM)

Autoimmune diseases are characterized by the inability of an individual's immune system to discriminate between 'self' and 'non-self' molecules. This may lead to an attack on self tissues or cells. To date many autoimmune disorders have been described with known or unknown etiology. Connective tissue diseases such as systemic sclerosis (SSc), systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), mixed connective tissue disease (MCTD), and myositis (idiopathic inflammatory myopathies or IIM) have no known cause, but they all are characterized by the presence of autoantibodies.

Myositis is characterized by progressive skeletal muscle weakness, elevated serum levels of muscle enzymes (e.g. creatine kinase, aldolase, lactate dehydrogenase) and distinct inflammatory features. Further clinical, immunological and pathohistological characteristics are used to distinguish between the different myositis subtypes which are polymyositis (PM), dermatomyositis (DM) and inclusion-body myositis (IBM) [1-3]. Depending on the study, incidence numbers of myositis patients vary from 1.0 to 6.3 per 100,000 persons [4]. The development of DM and PM can start in childhood and between 30 and 50 years, whereas IBM most frequently occurs in individuals of over 50 years. IBM occurs more often in elderly males (3:1), whereas DM and PM are more commonly found in females compared to males (2:1). Diagnostic factors in PM patients are characterized by a subacute onset of symmetrical proximal muscle weakness accompanied by $CD8^+$ T cells and macrophages that invade muscle fibers which express high levels of MHC class I molecules [5]. IBM patients develop slow and progressively distinct asymmetric proximal muscle weakness together with the infiltration of MHC class I expressing muscle fibers by $CD8^+$ T cells, similarly as seen in PM [6]. Moreover, in IBM myodegenerative processes are detected that are associated with vacuolisation of muscle fibers and accumulation of proteins, like amyloid-related proteins, β-amyloid precursor protein (APP), phosphorylated tau, and proteins involved in oxidative stress [7]. Individuals with DM contain different immunological factors, such as $CD4^+$ T cells, B cells and dendritic cells (DCs) in perivascular muscle infiltrates. Additionally, characteristic skin manifestations, such as Gottron's papules (60 – 80% of patients), heliotropic rash (50% of patients), 'shawl' sign and so-called 'mechanic hands' can also be detected in DM patients [8,9]. However, increasing knowledge about myositis heterogeneity may lead to a further subclassification of this disease, such as cancer-associated myositis, clinical amyopathic myositis, myositis overlap, necrotizing myositis, and childhood myositis.

Etiology of myositis

The current ideas on the development of autoimmune diseases emphasize a multifactorial background in the breaking of immunological tolerance. Several environmental factors, like smoking [10] and UV-light [11], as well as genetic factors [12] were shown to be involved in disease onset or progression. Additionally, in up to 80% of myositis patients so-called myositis-associated autoantibodies (MAA) or myositis-specific autoantibodies (MSA) directed to a variety of cellular components can be found [13,14]. MAA, such as anti-Ro52, anti-Ku and polymyositis-scleroderma overlap autoantibody (anti-PM/Scl) are not specific for myositis and also found in other rheumatic disorders. The MSA are highly specific for myositis and include anti-aminoacyl-tRNA synthetases (e.g. Jo-1, PL-7), anti-Mi-2 and anti-signal recognition particle (anti-SRP) antibodies (reviewed by [15]). Insight into the autoantibody repertoire of myositis patients is important to predict progression and outcome of the disease.

Muscle fiber necrosis in IIM is associated with

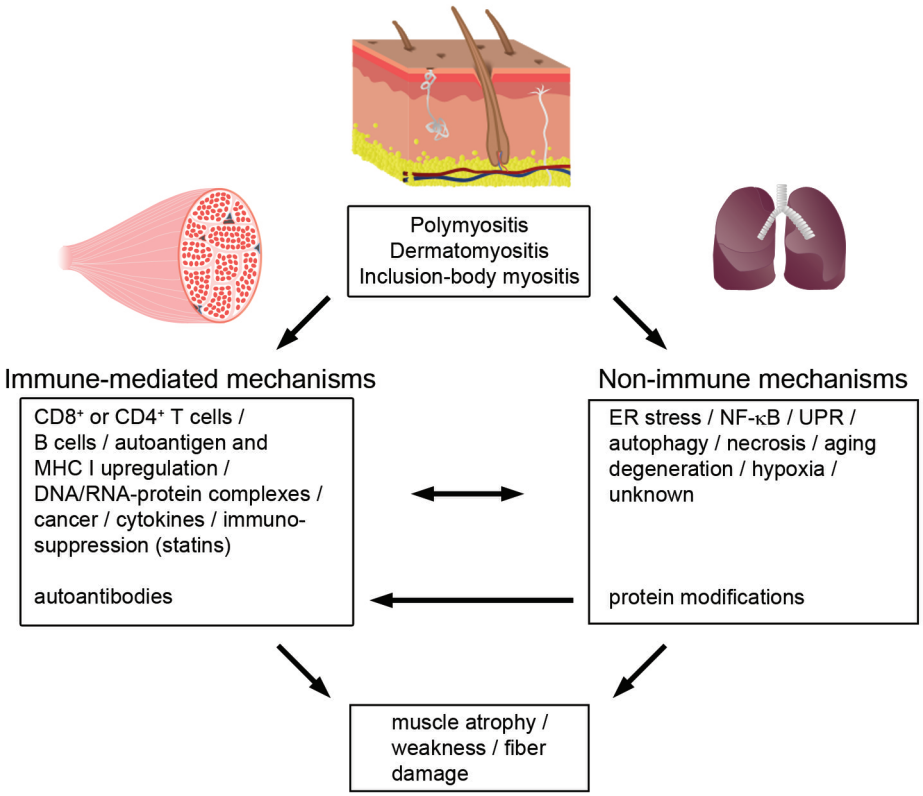

Fig. 1 Factors involved in the development of myositis.
Clinical manifestations of skeletal muscle, skin, and lung tissue are often found in individuals suffering from myositis. The development of this disease is believed to result from immune- or non-immune-mediated mechanisms and lead to skeletal muscle damage. Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; NF-κB, nuclear factor-κB; MHC, major histocompatibility complex. Adapted from [17]

mononuclear inflammatory cell infiltrates in skeletal muscle tissue. However, these inflammatory processes do not always correlate with the severity of disease. The mononuclear infiltrates mainly consist of B cells and T cells, although also macrophages, DCs and natural killer cells are found in inflamed muscle tissue [16]. Together with a diverse expression profile of cytokines and chemokines, a complex inflammatory milieu is commonly found in affected tissues of myositis patients. The inflammatory environment can stimulate cell death to occur locally. Indeed, loci of extensive necrotic cell death have been detected in myositis patients and $CD8^+$ T cells are found to surround non-necrotic muscle fibers (reviewed by [5]). Cell death has been associated with autoimmunity for many years. During cell death many (bio)chemical processes occur that post-translationally modify endogenous proteins. The protein modifications might be involved in breaking immunological tolerance and/or promote inflammation. Furthermore, many environmental factors (e.g. viruses, UV-light, smoking) as well as secreted molecules (e.g. perforin, granzyme B, nitric oxide ($NO^{\bullet}$)) have been described to stimulate cell death. In addition to the immune mediated responses mentioned above, evidence accumulates that also non-immune processes play a role in the pathogenesis of myositis. An excellent review by Henriques-Pons and Nagaraju discusses the endoplasmic reticulum (ER) stress response, nuclear factor-κB (NF-κB) signaling and autophagy as possible non-immune pathways by which muscle damage or autoimmunity might develop in myositis patients [17]. In addition, NF-κB is thought to connect both immunological and non-immunological pathways to muscle damage. How the combination of structural, immunological and environmental alterations relates to pathological processes involved in IIM remains, however, elusive (Fig. 1).

Cell death and autoimmunity

Cell death may occur as a response to physiological or pathological triggers encountered by a tissue or a cell. The criteria of the different types of cell death

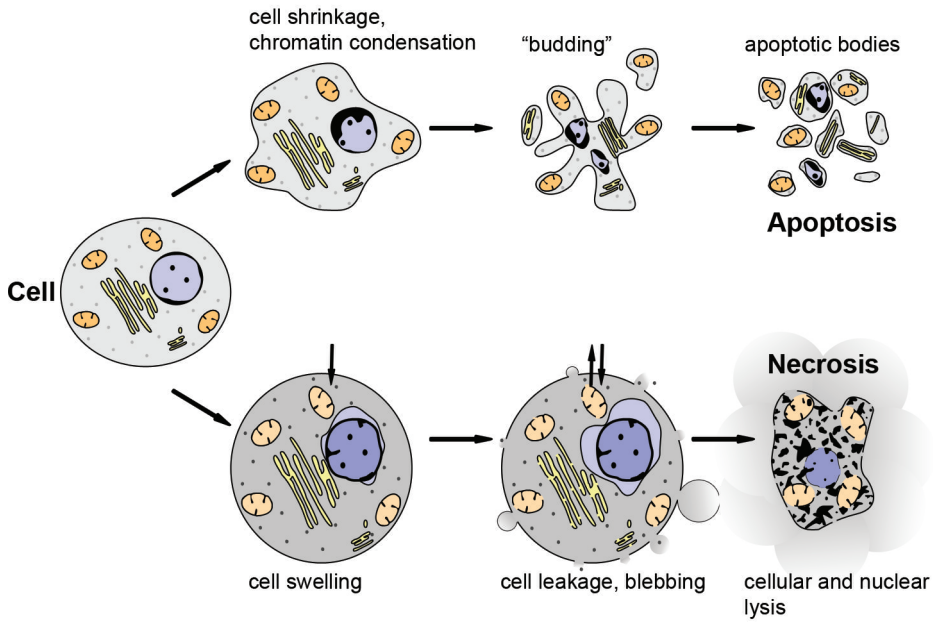

Fig. 2 Schematic representation of apoptotic and necrotic cell death.
Apoptosis: After the induction of apoptosis cells shrink and chromatin condenses, followed by controlled degradation of cell organelles and relocalization. Small amounts of cellular content, confined by a membrane, are released from the apoptotic cell ("budding"). The cells disintegrate to apoptotic bodies, which normally are cleared by neighbouring phagocytic cells without inducing inflammatory processes. Necrosis: Cells take in extracellular fluid and swell, which results in the loss of membrane integrity, leakage of cellular content and blebbing. Finally, cellular and nuclear lysis occurs. In addition, the release of its cellular content may trigger inflammation. Adapted from [75].

are often defined by morphological changes and the involvement of specific enzyme systems such as proteases and nucleases. There are two major mechanisms of cell death: apoptosis and necrosis. These two forms and their potential relationship with myositis will be introduced below (Fig. 2).

Apoptosis and myositis

Apoptosis, also called programmed cell death (PCD), is a well studied phenomenon and associated with many physiological processes, such as embryonic development, immune system regulation (induction of tolerance) and anti-cancer mechanisms [18-20]. This ATP-dependent mechanism of cell death results in the controlled degradation of the cellular organization, which is a prerequisite for the appropriate clearance of the dying cell by surrounding cells and phagocytes. Apoptosis is an energy-consuming process that leads to the well-defined compartmentalization of cellular structures found during the dying process. Cell shrinkage, loss of membrane asymmetry, chromatin condensation, DNA fragmentation, and membrane blebbing are distinct changes that occur when cells undergo apoptosis. The apoptotic blebs/bodies contain many cellular proteins that might stimulate the autoreactive T cells and/or B cells when they are released from these vesicles. However, these confined structures are normally cleared efficiently by neighbouring phagocytic cells.

Two main apoptotic pathways, the extrinsic and intrinsic pathways, regulate the induction of PCD, both with slightly different activation modes. The FasL and TNF-α related apoptosis-inducing ligand (TRAIL) stimulate cell surface receptors of the TNF receptor (TNFR) family, like TNFR, Fas and TRAIL-R, which initiates the extrinsic pathway. Activation of these receptors can result in various cellular responses, such as proliferation, differentiation and cell death. The formation of a death-inducing signaling complex (DISC) induces apoptosis by the recruitment and activation of initiator caspase-8 and caspase-10, which further downstream activate executioner caspases that are also involved in the intrinsic pathway [21].

DNA damage and cytotoxic insults activate the intrinsic pathway by antagonizing the anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-Xl, and Bcl-w) that maintain mitochondrial integrity [22]. As a result, the pro-apoptotic Bcl-2-like proteins (e.g. Bax, Bak, Bad, and Bim) release cytochrome c from the mitochondria. Cytochrome c is needed for the formation of the apoptosome that activates the initiator caspase-9. Finally, caspase-9 will cleave pro-caspases-3, -6, and -7, which are responsible for the

Table 1. Reactive oxygen and reactive nitrogen species

Reactive oxygen species (ROS)	Reactive nitrogen species (RNS)
Superoxide anion ($O_2^{\bullet -}$)	Nitric oxide ($NO^{\bullet}$)
Hydroxyl radical ($OH^{\bullet}$)	Nitric dioxide ($NO_2^{\bullet}$)
Peroxyl radical ($ROO^{\bullet}$)	Peroxynitrite ($ONOO^-$)
Hydrogen peroxide (H_2O_2)	Peroxynitrous acid (ONOOH)
Hypochlorous acid (HOCl)	Alkylperoxynitrite (ROONO)

substrate cleavage typical for apoptotic cell death [23,24]. Post-translational modifications, such as the cleavage of proteins, are essential for the induction of apoptosis, but may also be involved in the initiation of pathological events, such as autoimmunity.

Both pro-apoptotic and anti-apoptotic Bcl-2 proteins appear to be associated with the development of or protection against rheumatoid arthritis (RA). Anti-apoptotic Mcl-1 expression was increased in RA synovial macrophages and fibroblasts and correlated with the degree of inflammation [25,26]. The increased Mcl-1 levels reduced apoptotic cell death in the synovium, which suggests that it is a critical molecule for cell survival and a potential therapeutic target in RA. In addition, Bim and Bid, potent initiators of apoptosis in multiple cell types, have been shown to be involved in joint cellularity and local inflammation of RA patients by maintaining apoptotic homeostasis [27]. Altered levels of these pro-apoptotic and anti-apoptotic molecules are also found in other autoimmune disorders, like myositis. The high incidence of cytotoxic T lymphocytes (CTLs), which are capable of inducing apoptosis, suggest an important role for PCD in myositis. In addition, Sugiura and colleagues describe fiber apoptosis as a result of the binding of mononuclear cells, expressing FasL, to Fas-containing muscle fibers [28]. However, several other groups were unable to confirm these findings [29-32]. These contradictory results might be explained by the upregulated expression of anti-apoptotic proteins that counteract these pro-apoptotic signals. Recently, Kondo *et al.* emphasized the pro-inflammatory cytokine microenvironment to promote muscle fiber apoptosis after Fas/FasL interaction [33]. Indeed, many cytokines and chemokines are found to be upregulated in IIM patients (reviewed by [16]). One candidate might be the DNA-binding protein high mobility group box protein 1 (HMGB1), which under physiological conditions is involved in transcription, but also can act as a pro-inflammatory molecule when released during cell death. HMGB1 is actively released from macrophages/monocytes in PM/DM patients and induces the expression of TNF-α and IL-1, which are both pro-inflammatory cytokines [34]. Moreover, HMGB1 has been shown to induce the expression of MHC class I molecules in murine muscle fibers and was suggested to be involved in muscle dysfunction during the first stages of IIM [35]. In addition, recently elevated numbers of so-called micro-particles, which are involved in intercellular communication, were found in autoimmune disorders such as PM/DM [36,37]. However, the actual role of these particles, whether it is induction, amplification or counteracting the autoimmune response, is not clear yet.

Besides immunological triggers, environmental factors are also known to play an important role in the development of autoimmunity. Rosen *et al.* reported that UV-B irradiated keratinocytes develop apoptotic manifestions leading to distinct subcellular localization of several autoantigenic proteins, including Jo-1, Mi-2, Ro52, and Ku [38]. Cytoplasmic or nuclear antigens eventually can be found in apoptotic blebs or bodies of the dying cell. UV-B exposure is known to increase the formation of intracellular reactive oxygen species (ROS), which are able to oxidatively damage a (selective) number of biomolecules and can result in the loss of cell function and/or death [39,40]. Moreover, D'Emilio and colleagues demonstrated the occurrence of apoptotic changes in UV-B irradiated cultured myoblasts and myotubes due to elevated ROS levels [41]. The overproduction of ROS in skeletal muscle cells might correlate with immune responses against muscle fibers found in myositis. How ROS and derivates might be implicated in the development of myositis will be discussed later. In addition, smoking has been suggested to stimulate apoptosis in inflammatory rheumatic disorders [10]. It should, however, be noted that indisputable evidence for the association of apoptotic muscle fibers with the development of myositis has not been produced yet.

Andrade and colleagues detected a caspase-independent ability of granzyme B (GrB) to cleave several downstream death substrates [42]. Further studies expanded the number of GrB substrates and revealed unique fragments from autoantigenic proteins that are not found in other forms of cell death [43]. A putative substrate for GrB is the histidyl-tRNA

synthetase (HisRS), also designated Jo-1 protein, which is the major autoantigen in PM/DM patients [44]. The resulting N-terminal part of Jo-1, but also full-length Jo-1, stimulated primary $CD4^+$ T cells, or $CD8^+$ lymphocytes, as well as immature dendritic cells, which may perpetuate the development of myositis [45,46].

Necrosis and myositis

Necrotic cell death was considered an uncontrolled form of cell death for years. This might be true after severe physical damage, but increasing evidence supports the existence of a regulated caspase-independent pathway as well [47]. Furthermore, the release of intracellular proteins to the extracellular environment was thought to elicit pro-inflammatory stimuli that could attract destructive inflammatory cells to the site of tissue damage or activate local antigen-presenting cells. However, it is clear that these cells are not only involved in tissue destruction but also are needed to battle potential infections, to remove cellular debris and/or to participate in the regeneration of damaged tissue. Infiltrating macrophages appear to be involved in the activation of satellite cells that are needed to regenerate injured muscle tissue [48-51]. Necrosis-induced morphological changes are distinct from the structures found in apoptotic cells and include cell and organelle swelling, with subsequent loss of cell membrane integrity and release of the cellular content into the surrounding extracellular environment. Several proteins/enzymes under distinct conditions have been shown to play a role in the initiation of necrosis, such as TNFR1, RIP1, TRADD and TLRs [21]. These molecules work on downstream factors such as ceramide, reactive oxygen species (ROS), phospholipases, and calpains that may lead to necrotic cell death. However, a lot of mechanisms and players in this process remain as yet unknown.

There is only little experimental evidence concerning the contribution of necrosis to the development of autoimmune disorders. However, necrotic loci and the infiltration of immune cells into non-necrotic sites are important parameters for the diagnosis of myositis. Autoaggressive $CD8^+$-cytotoxic T cells are found to invade MHC class I expressing muscle fibers in PM and IBM patients. These T cells are thought to release perforin granules, which result in muscle fiber necrosis rather than apoptosis, possibly by the overexpression of anti-apoptotic molecules described in the previous section. Complement deposits induce capillary necrosis, perivascular inflammation, and muscle fiber destruction in DM patients (reviewed by [5]). The tissue damage, as a consequence of necrosis, results in the development of muscle atrophy and to a decreased number of blood vessels leading to tissue ischemia in myositis patients. Whether these clinical manifestations are cause or consequence of the disease has yet to be established.

Oxidative stress and autoimmunity

Reactive oxygen species are chemically-reactive molecules containing oxygen, such as oxygen ions and peroxides. ROS can be generated in the mitochondria as a by-product of the electron transport chain, which is mediated by complexes that are involved in the cellular energy metabolism, generating energy-rich molecules such as ATP, NADH, and $FADH_2$. ROS can also be generated by NADPH oxidase, a plasmamembrane-bound enzyme complex that catalyzes the electron transfer across the membrane from NADPH to O_2 within the extracellular space or within specialized compartments (e.g. phagosomes). This mainly results in the production of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Both molecules are implicated in physiological responses, such as defense mechanisms against pathogens (e.g. respiratory burst by macrophages) or cell signaling (reviewed by [52]). In addition, several other enzymes in many different cell types are able to produce ROS, including xanthine oxidase, cyclo-oxygenases, myeloperoxidases, as well as cytochrome P450-based enzymes. The free radicals produced by these enzymes are $O_2^{\bullet -}$, hydroxyl radicals ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$), whereas non-free radicals include H_2O_2 and hypochlorous acid (HOCl). Besides ROS also several intermediates of reactive nitrogen species (RNS) are generated and contribute to physiological functions, such as vasorelaxation and neurotransmission [53]. These intermediates are derived from nitric oxide ($NO^{\bullet}$) that is synthesized during the conversion of free L-arginine to L-citrulline by nitric oxide synthetases (NOS) [54]. The RNS comprise free radical nitric dioxide ($NO_2^{\bullet}$), $NO^{\bullet}$ and various non-free radicals such as peroxynitrite ($ONOO^-$) and protonated forms including peroxynitrous acid (ONOOH), and alkylperoxynitrite (ROONO) (Table 1).

The radicals most often found participating in physiological processes are $O_2^{\bullet -}$, H_2O_2 and $NO^{\bullet}$. In order to maintain the cellular redox balance and to prevent cell damage, various anti-oxidant systems, such as glutathione (GSH), vitamins and enzymes (e.g. catalase, peroxidases, and superoxide dismutases), continuously remove or neutralize ROS/RNS [55]. The low physiological concentrations of free radicals are sufficient to exert their function.

However, next to cellular processes also xenobiotics, drugs, cytokines and environmental factors (such as UV-light, and smoking) can increase ROS/RNS production. Under pathophysiological conditions ROS/RNS production might exceed the endogenous detoxification and/or utilization capacity which may result in oxidative or nitrosative stress depending on its chemical composition or environment.

This suggests a dual role for ROS/RNS: 1) physiological (protective) functions that are summarized above, and 2) harmful (destructive) effects resulting from oxidative or nitrosative stress [56]. The stress responses describe conditions in which radicals mediate negative effects in vivo. Both ROS and RNS are able to modify biomolecules such as DNA, proteins, lipids and carbohydrates when produced at non-physiological levels and such modifications may lead to loss of cell function and apoptotic or necrotic cell death [57-60]. Certain radical-induced modifications are associated with specific radical – biomolecule interactions and are highly dependent on the differences in ROS/RNS half-life, reactivity, and chemical composition. The destructive nature of ROS/RNS has been emphasized to play a role in the aging process, in carcinogenesis and in several pathologies including cardiovascular disorders, neurodegenerative disorders, RA, and SLE [61-63].

The main target tissue of the immune system in myositis, the skeletal muscle fibers, is of particular interest with respect to oxidative and nitrosative stress effects because of its continuous exposure to ROS/RNS. Skeletal muscle fibers consume relatively high amounts of ATP to perform their function and therefore are depending on a well performing oxidative metabolism and anaerobic metabolism. During in vitro exercise studies, skeletal muscles show increased levels of free oxygen radicals that contribute to oxidative stress and stimulate peroxynitrite formation with $NO^{\bullet}$ [64,65]. Studies with cultured skeletal myoblasts and T-cells also led to the detection of cell death upon treatment with low doses of H_2O_2 [66,67]. Abnormal cytochrome c oxidase activities are emphasized to result in mitochondrial abnormalities, muscle weakness and wasting that are found in muscle fibers of DM and IBM patients [68,69]. Moreover, studies of de Paepe *et al.* showed elevated expression levels of inducible NOS in PM and IBM patients in comparison with healthy controls [70]. In addition, markers of $NO^{\bullet}$ production were shown to be elevated in IIM patients compared to controls, which suggests increased NOS activity in these patients [71]. Recently, He and colleagues reported the activation of the ER stress response upon the accumulation of ROS in the ER of retinal pigment epithelial cells [72]. A similar process might be responsible for the activation of ER stress response and increased chaperone expression in myositis patients [73,74]. Taken together, the results of all of these studies are consistent with a role of ROS/RNS in the pathophysiology of myositis. However, further studies will be required to clarify whether ROS/RNS directly contribute to the development of myositis, or whether the elevated levels of ROS/RNS are an epiphenomenon resulting from the cellular infiltrates found in IIM patient muscle tissue.

Outline of this thesis

The studies described in this thesis were initiated to obtain more insight into ROS/RNS-induced protein modification of myositis autoantigens and their association with the autoimmunological features of this disease. As described above, ROS/RNS have many physiological and pathological functions and are capable of activating cell death mechanisms as well as modifying biomolecules such as DNA, lipids, carbohydrates, and proteins via (in)direct mechanisms. The results of these studies were expected to provide clues for the contribution of ROS/RNS to post-translational modification-mediated loss of immunological tolerance against myositis-specific autoantigens.

In Chapter 2 the diagnostic and research methodologies that are currently used to identify and detect myositis-specific biomarkers, which are essential for an adequate diagnosis, prognosis, and treatment, are introduced. In addition, the established and recently identified myositis-specific autoantibodies and their targets are discussed, as well as their importance for the classification of myositis subtypes and/or pathogenesis.

The modification of several autoantigenic proteins under oxidative stress conditions is presented in Chapter 3. The direct and indirect effects and nature of oxidative stress-associated free radicals were addressed in cells and recombinant proteins, and also the identity of the most relevant ROS species are presented using specific generators of ROS. Chapter 4 describes novel oxidative stress-induced post-translational modifications of the major myositis autoantigen Jo-1 (HisRS), which were identified by immunoblotting assays and characterized by mass spectrometry-based methods. The effects of the ROS-induced post-translational modifications on the tRNA aminoacylation activity of Jo-1 and its recognition by anti-Jo-1 antibodies in patient sera are described as well. In the studies presented in Chapter 5 the

redox-sensitive dimerization of the Jo-1 molecule is documented. The modifications involved in HisRS homodimerization, both *in vitro* and in living cells, and their effects on its enzymatic activity are elucidated by mass spectrometry, computational modeling, and functional analysis. The studies described in Chapter 6 allowed the identification of a distinct Jo-1 modification that was observed to occur in RNS-stressed cells. Under these conditions, endogenous Jo-1 as well as the ectopically expressed wild-type Jo-1 protein appeared to form covalent dimers, not mediated by disulfide bridges. The effects of amino acid substitution mutants on dimerization, the enzymatic activity and autoantigenicity were studied as well.

In Chapter 7 the identification and characterization of the first IBM-specific autoantibody biomarker is reported. This was only possible because human skeletal muscle extracts were used as a source of potential autoantibody targets. The methods used to identify these IBM-specific autoantibodies emphasize the importance of the source material used in studies aimed at new biomarkers in autoimmunity.

Finally, in Chapter 8 the experimental results of the studies described in this thesis are discussed in a more general context.

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Chapter 2

Myositis-specific antibodies (MSA): detection and clinical associations

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Abstract

In recent years, the detection and characterization of (novel) autoantibodies is becoming increasingly important for the early diagnosis of autoimmune diseases. The idiopathic inflammatory myopathies (IIM, also indicated with myositis) are a group of systemic autoimmune disorders that involve inflammation and weakness of skeletal muscles. One of the hallmarks is the infiltration of inflammatory cells in muscle tissues. A number of myositis-specific autoantibodies have been identified and these may be associated with distinct IIM subclasses and clinical symptoms. Here, we review all myositis-specific autoantibodies identified today as well as their target proteins, together with their clinical associations in IIM patients. Post-translational modifications that might be associated with the generation of autoantibodies and the development of the disease are discussed as well. In addition, we describe well established autoantibody detection techniques that are currently being used in diagnostic laboratories, as well as novel multiplexed methods. The latter techniques provide great opportunities for the simultaneous detection of distinct autoantibodies, but may also contribute to the identification of novel autoantibody profiles, which may have additional diagnostic and prognostic value. The ongoing characterization of novel autoantibody specificities emphasizes the complexity of processes involved in the development of such autoimmune diseases.

Autoantibodies in idiopathic inflammatory myopathies

Autoantibodies directed against a variety of autoantigens are strongly associated with systemic autoimmune diseases. Within the heterogeneous group of connective tissue diseases (CTD) a wide range of nuclear and cytoplasmic autoantigenic targets have been described. However, it is still unclear why autoantibodies directed against these autoantigens are formed and whether they participate in pathological processes during disease development and/or progression. Post-translational modifications (PTMs) have been hypothesized to generate novel epitopes in antigenic proteins. The cross-presentation of these post-translationally modified epitopes might break immunological tolerance and initiate the development of an autoimmune response resulting in autoantibody formation.

The idiopathic inflammatory myopathies (IIM) are a group of disorders characterized by inflammation and weakness mainly of the muscles closest to the trunk of the body (proximal muscles). These disorders include polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM). IIM may be associated with inflammation in other organs, including the joints, heart, lungs, intestines and skin. In IIM, like in other systemic autoimmune diseases, distinct clinical phenotypes can be correlated with specific autoantibody targets in patients, which emphasizes the clinical relevance of these biomarkers [1]. Autoantibodies found in IIM patients can be divided into myositis-associated autoantibodies (MAA), which are not specific for the disease and are also found in other rheumatic disorders, and myositis-specific autoantibodies (MSA) which are found primarily in patients with IIM. Tables 1 and 2 show a comprehensive overview of the autoantigens recognized by MSA and MAA together with their function. In this review we will describe the most prevalent MSA in more detail, their detection and the clinical manifestations associated with them.

Autoantibody detection assays

The development of less invasive and more advanced imaging tools in order to discriminate between the clinical characteristics of various inflammatory myopathy subtypes, contributed greatly to the often difficult diagnosis of these diseases. The application of advanced techniques such as magnetic resonance imaging (MRI), X-ray computed tomography (CT), positron emission tomography (PET), and ultrasonography can also increase the sensitivity of detecting IIM prior to muscle biopsy [2]. In addition, several serological markers in IIM which can be specific for certain IIM subtypes or be helpful in evaluating disease progression have been detected in the last decades. These biomarkers mostly are autoantibodies directed to one or more cellular components. The fact that only a limited number of proteins appear to be targets of autoantibodies suggests that distinct processes are involved in their generation.

A variety of techniques are available to detect the various autoantibodies for research or routine diagnostics, all with their distinct sensitivity and specificity traits. Conventional methods such as indirect immunofluorescence, immunoprecipitation, immunodiffusion and counterimmuno-electrophoresis, or immunoassays such as the enzyme-linked immunosorbent assay (ELISA) and immunoblotting are already in use for many years. In most cases these techniques are suitable for the detection of a single autoantibody specificity. In contrast, a new generation of assays (so-called multiplexed analysis techniques) allows the simultaneous detection of several autoantibody specificities [3, 4]. The latter techniques probably will dominate serological profiling in diagnostic laboratories in the future, provided that validation and reproducibility are ensured.

Immunofluorescence

Several antinuclear antibodies (ANA) are still being detected by indirect immunofluorescence on HEp-2 cells or tissue substrates. Some ANA-positive IIM sera may show a particular staining pattern that can be correlated with a distinct autoantigen. However, this technique is laborious and needs specific technical skills to perform. It is therefore less convenient for present day diagnostic analyses.

Immunoprecipitation, ELISA and immunoblotting

The most unambiguous way to determine the presence of autoantibodies against a particular autoantigen is the detection of an autoantibody by virtue of its binding to the antigen in its fully native state. Although the immunoprecipitation (IP) method is the most obvious choice, this technique is not widely applied because it is laborious, expensive and requires specific technical skills. Radiolabeled cell extracts are often used as substrates for immunoprecipitation assays and subsequent mass-spectrometry analysis can characterize the specific targets [5, 6] (Fig. 1). However, the antigen to be detected may be associated with other (non-antigenic) proteins and as a consequence the IP procedure often leads to a complex pattern of precipitated proteins of which

Table 1. Myositis-specific autoantibodies and their functional activities

Autoantibody	Autoantigen	Molecular Mass (kDa)	Function of autoantigen
Myositis-specific autoantibodies (MSA)			
Anti-aaRS			
Anti-Jo-1/PL1	HisRS	54	Aminoacylation of tRNAs
Anti-PL7	ThrRS	80	
Anti-PL12	AlaRS	106	
Anti-EJ	GlyRS	75	
Anti-OJ	IleRS	150	
Anti-KS	AsnRS	65	
Anti-Ha	TyrRS	58	
Anti-Zo	PheRS	57/66	
Anti-tRNA	$tRNA^{His}$		Translation
	$tRNA^{Ala}$		
Anti-Mi-2	Mi-2α	220	Transcription regulator & nucleosome remodelling
	Mi-2β	218	
Anti-SRP	SRP54	54	Protein translocation to the ER
	SRP68	68	
	SRP72	72	
Anti-p155/140	Tif1-γ	155	Ubiquitination
	unknown	140	n.d.
Anti-CADM-140	MDA5	117	Innate immunity (RNA sensor)
Anti-SAE	SAE1	38	Sumolytion
	SAE2	72	
Anti-p140	NXP-2	107	RNA binding & nuclear transcription

not determined: n.d.

only one or a few proteins are truly autoantigenic. Nevertheless, detection of autoantigenic biomarkers in IIM by immunoprecipitation techniques is still widely used.

The first high-throughput assays such as ELISA increased the number of patient sera that could be analysed compared to the previously mentioned conventional assays. However, purification of the antigen and use of the correct reagents has to be monitored to warrant an optimal specificity [7, 8]. In addition, at least some conformational epitopes might be lost, because the antigen is bound to the ELISA plate. Immunoblotting (IB) analysis, in addition to ELISA, may increase the specificity of the signal detected in the immune assays. However, IB may allow the detection of autoantibodies only to linear epitopes, because the antigen is completely denatured during preparation of the blot. This might decrease sensitivity of the IB method as compared to IP and ELISA. As a consequence only the ELISA method is widely used in routine laboratories where large numbers of sera have to be tested.

Multiplex assays

A number of multiplex-based assays have been developed during the years, with distinct methodological differences. In this review we will discuss the high-throughput-based micro-array format, described in this paragraph, and a macroscopic assay that has been developed for the simultaneous detection of multiple autoantibodies in IIM sera, the line-blot, which is described separately in the next paragraph. Multiplex assays are able to screen a single sample of blood or other biological fluid for an array of autoantibody specificities simultaneously. In this way a patient-specific autoantibody profile can be made. Future prospects suggest that antigen/patient-specific therapies might be developed from these patient-specific autoantibody profiles, which might prevent disease progression, remission or relapse

Table 2. Myositis-associated autoantibodies and their functional activities

Autoantibody	Autoantigen	Molecular Mass (kDa)	Function of autoantigen
Myositis-associated autoantibodies (MAA)			
Anti-PM/Scl	PM/Scl-75	75	RNA processing & degradation
	PM/Scl-100	100	
Anti-Mas	Serine-tRNA^{Sec} protein complex	48	Selenocysteine incorporation
Anti-Ro/SS-A			
Anti-Ro52	Ro52	52	
Anti-SS-A	Ro60	60	RNA quality control
Anti-La/SS-B	La	48	RNA binding & Pol III transcription
Anti-RNP	U1A	34	Pre-mRNA splicing
	U1C	22	
	U1-70k	70	
Anti-Wa	Peptide	46	unknown
Anti-PMS	a.o. PMS1, PMS2	n.d.	DNA binding protein complex involved in DNA repair
Anti-Ku	Ku70	70	DNA dependent protein phosphorylation
	Ku80	80	
Anti-Fer	eEF1		Translation
Anti-KJ	Peptide	30/34	Translation factor
Anti-56k	Nuclear RNP	56	unknown

polymyositis, scleroderma overlap (PM/Scl), ribonucleoprotein (RNP), postmeiotic segregation (PMS), eukaryotic elongation factor (eEF1), not determined: n.d.

over time. The basic principles of two main types of micro-array-based multiplex assays will be discussed below.

Solid surface-based autoantigen microarrays contain immobilized proteins or other biomolecules in predetermined positions on a solid surface. Interactions between the immobilized antigens and molecules in the serum sample such as (labeled) antibodies can be detected by fluorescence-based procedures [4, 9]. Such antigen arrays have already been used to screen the autoantibody profiles in autoimmune diseases, IgE reactivity in allergy, and the immune response to infections, vaccination, and cancer reviewed by [4]. However, this technique is based on commercialized or home-made microarrays that depend on specific technical expertise and equipment (Fig. 2). In order to simplify technical aspects as much as possible so-called *addressable-bead autoantigen microarrays* have been developed [10, 11]. Individual antigens of interest are chemically coupled to beads of different colours. Subsequently, sera or other biological fluids can be analyzed in a microtiter well containing a bead mixture as described by [3, 12]. One laser will measure the colour of the specific antigen-coupled bead, whereas a second laser determines the presence and quantity of a fluorochrome-coupled secondary antibody bound to the bead (Fig. 3). Both autoantigen microarray techniques have advantages over the conventional techniques in terms of reduced sample volumes, enhanced sensitivity, automation and increased numbers of samples that can be tested. The sometimes imperfect specificity and reproducibility of these methods can be obstacles for their implementation in routine diagnostics.

Line-blot assay

The line-blot assays, or so-called line immunoassays (LIAs), are based on immunoblotting procedures that spot purified antigens on protein binding membranes without the need of gel electrophoresis (Fig. 4). The laborious purification procedure of native antigens is being replaced by the more reproducible production of highly purified recombinant antigens or synthetic peptides. These developments contribute to the increased sensitivity and specificity of commercially

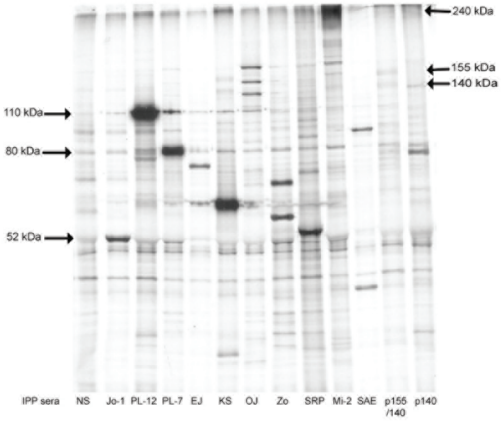

Fig. 1 Immunoprecipitation assay with myositis-specific autoantibodies. Polypeptides immunoprecipitated from ^{35}S-methionine-labelled K562 cell extracts by antibodies from patient sera were separated by SDS-polyacrylamide gelelectrophoresis and visualized by autoradiography. Sera used for immunoprecipitation included serum from a healthy individual (NS), anti-Jo-1, anti-PL-12, anti-PL-7, anti-EJ, anti-KS, anti-OJ, anti-Zo, anti-SRP, anti-Mi-2, anti-SAE, anti-p155/140, anti-p140 (reproduced from [6], by permission of The British Society for Rheumatology).

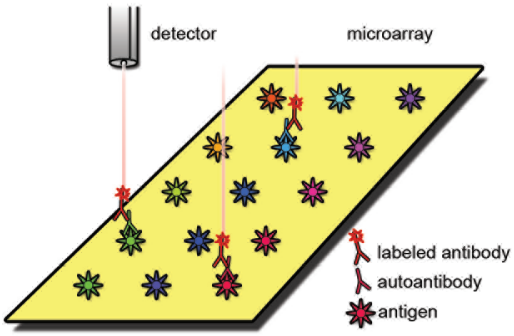

Fig. 2 Schematic representation of the *solid surface-based autoantigen array.*
Autoantigenic molecules are immobilized at defined positions on a solid surface. Binding of autoantibodies in patient sera to the immobilized autoantigens can be detected via fluorescently labelled secondary antibodies.

available line-blots. Several LIAs of different manufacturers have recently been clinically validated in multicenter studies, and the results indicate that LIAs are becoming a suitable alternative to the more costly and complex techniques sometimes used in diagnostic laboratories reviewed by [13, 14].

Myositis-specific autoantibodies

Aminoacyl-tRNA synthetases

Function

An efficient and reliable transcription and translation of the genetic code is essential for cell survival. Genes are transcribed into messenger RNA (mRNA), which contains a string of nucleotide triplets, called codons. During translation every codon is translated into one particular amino acid (aa) with the help of transfer RNAs (tRNAs) (Fig. 5). The mRNA sequence is thus converted into a chain of amino acids that makes up a functional polypeptide. The tRNAs involved are specific for both the amino acid and the mRNA codon. The amino acids are coupled to their cognate tRNA via an esterification reaction that is catalyzed by specific aminoacyl tRNA synthetases (aaRSs) in a two-step reaction:

$$\text{aaRS} + \text{aa} + \text{ATP} \longleftrightarrow \text{aaRS*aa~AMP} + \text{PPi}$$

$$\text{aaRS*aa~AMP} + \text{tRNA} \longleftrightarrow \text{aaRS} + \text{aa-tRNA} + \text{AMP}$$

Most of the twenty canonical amino acids are recognized by a single aaRS, with the exception of GluRS and ProRS in higher eukaryotes [15, 16]. The aminoacylated-tRNA, or so-called charged tRNA, is used by the ribosome to incorporate the appropriate amino acid into the growing peptide chain.

In addition to the well-known function of aaRSs described above, a diverse array of other biological functions has been described as well. These include amino acid biosynthesis, DNA replication, RNA splicing, cell cycle control, and apoptosis (reviewed by [17, 18]).

Depending on highly conserved sequence motifs and aminoacylation acitivities, the aaRSs can be divided into two classes, Class I and Class II, and three subclasses a, b, and c (Fig. 6). The catalytic domain of Class I aaRSs consists of a typical Rossmann fold (nucleotide binding domain) and two highly conserved sequence motifs whereas the Class II aaRSs are characterized by an anti-parallel ß-strand surrounded by α-helices, and three conserved sequence motifs [19]. Additionally, Class I aaRSs mainly exist as monomeric or dimeric complexes and aminoacylate the 2'-hydroxyl group of the terminal ribose of tRNA. Class II aaRSs usually are dimeric or tetrameric structures and aminoacylate the 3'-hydroxyl group of the terminal ribose. The only exception is the PheRS that belongs to the Class II aaRSs but nevertheless aminoacylates the 2'-OH of the ribose. Higher eukaryotic cells have been shown to contain a high molecular mass multi-synthetase complex (MSC), which contains nine aaRSs and three accessory proteins (Fig. 6). The MSC is thought to promote protein synthesis, proofreading activity, and serve as a reservoir of regulatory molecules that are involved in functions other than aminoacylation [20, 21].

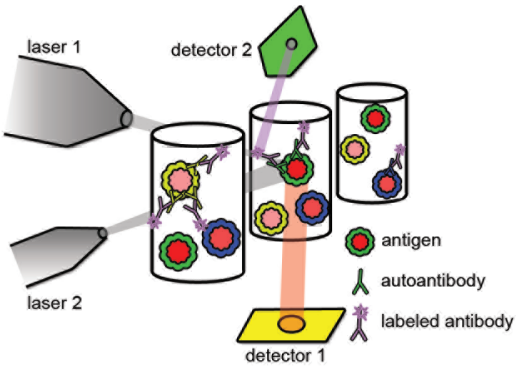

Fig. 3 Schematic representation of a *addressable-bead autoantigen immunoassay.*
Purified autoantigens are coupled to differentially labelled microbeads that can be detected by illumination with a laser. Patient sera can be incubated with mixtures of beads each coated with a different autoantigen. One laser is used to identify the specific antigen coupled to each bead based upon the fluorescent properties of the bead, and a second laser is used to determine the binding of autoantibodies to the beads after incubation with secondary antibodies conjugated to a distinct fluorophore.

Potentially disease-related PTMs

Three aaRSs (HisRS, AlaRS, and IleRS) have been described as substrates for the serine protease Granzyme B (GrB), which is present in granules of cytotoxic T-lymphocytes (CTLs) or natural killer (NK) cells and plays an important role in granule exocytosis-induced cytotoxicity [22, 23]. The exposure to GrB leads to unique fragments of autoantigens, which are not detected during other forms of cell death [24]. Indeed, almost all IIM autoantigens (except SSA/Ro52) can be cleaved by GrB, in contrast to non-autoantigenic proteins, which are either not cleaved or cleaved at sites also used by caspases [24]. The fragments generated by GrB, which are believed to contain new and unique epitopes, may help in breaking tolerance against the autoantigens when they are encountered by the immune system. The cleavage products as well as the full length autoantigenic HisRS and AsnRS have been described to exert chemotactic properties through their interaction with the chemokine receptors CCR3 or CCR5, in contrast to the non-autoantigenic AspRS and LysRS [25, 26]. Levine and colleagues found a proteolytically sensitive HisRS conformation in normal lungs compared to muscle tissue and proposed the lung as a target site where initiation and propagation against HisRS may take place [27]. However, the GrB-induced aaRS fragmentation and the subsequent dendritic cell-driven autoreactive $CD4^+$ T cell activation have not been demonstrated in IIM patients yet.

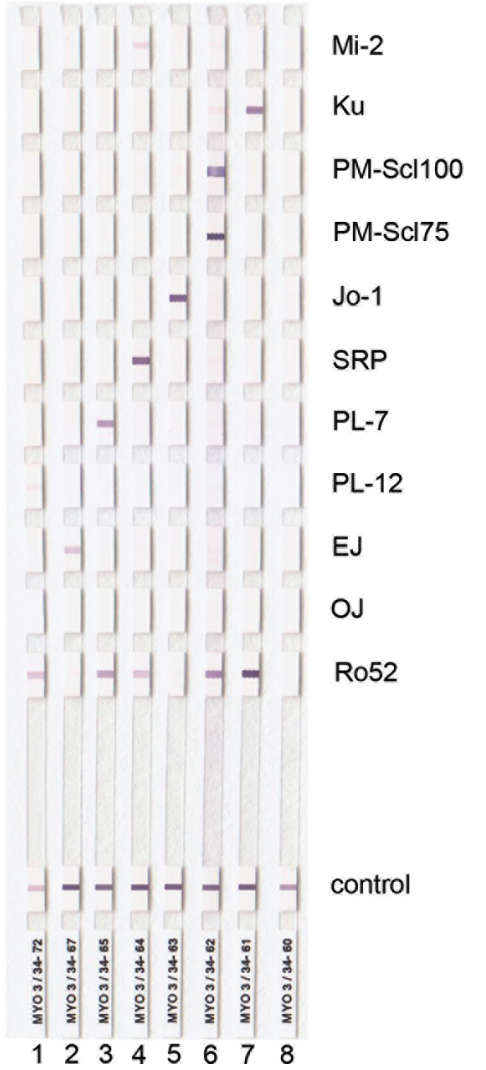

Fig. 4 Line-blot for autoantibody detection in IIM sera.
Strips (vertical) containing a series of recombinant IIM-related autoantigens were incubated with seven IIM patient sera (1-7) and a control serum (8). Antibody binding was visualized using the protocol provided by the manufacturer. The strips were incubated with sera containing the following autoantibodies (1) anti-PL12, (2) anti-EJ, (3) anti-PL7, (4) anti-SRP and anti-Mi-2, (5) anti-Jo-1, (6) anti-PM-Scl75 and anti-PM-Scl100, (7) anti-Ku.

An additional cleavage product of HisRS has been described by Ramirez-Sandoval and colleagues upon the induction of apoptosis in HEp-2 cells by camptothecin [28]. Also TyrRS, and its close homologue TrpRS, are both described to stimulate chemotactic activities as well as angiogenesis in vitro upon cleavage [29, 30]. The exposure to reactive oxygen and/or nitrogen species (ROS/RNS) may also lead to post-translationally modified biomolecules and result in the loss of immunological tolerance.

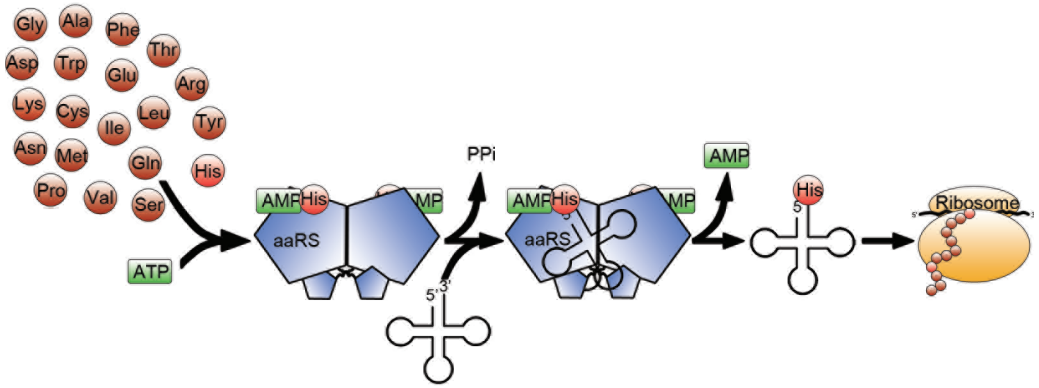

Fig. 5 Aminoacylation of tRNA by histidyl-tRNA synthetase.
The HisRS, as well as other aaRSs, catalyze the ATP-dependent esterification reaction that is needed to couple an amino acid to its cognate tRNA. Subsequently, the aminoacylated-tRNA can be used in translation. *AMP:* adenosine monophosphate, *ATP:* adenosine triphosphate, *PPi*: inorganic phosphate.

Interestingly, increased levels of ROS/RNS have been reported to be present in inflammatory autoimmune diseases [31, 32]. We have observed selective oxidative modifications in HisRS, but the significance of these for IIM remains to be established (Van Dooren *et al.*, manuscript submitted for publication).

Antibodies to aaRSs and their clinical associations

Autoantibodies directed to aminoacyl-tRNA synthetases are the most commonly detected MSA in adult IIM patients. They can be found in about 40% of individuals with IIM and in lower frequencies in juvenile IIM patients [33]. In addition, autoantibodies against the cognate tRNAs have been found [34, 35]. Most anti-aminoacyl-tRNA synthetase antibodies (anti-aaRS) are directed against the Class II aaRSs, including PL1/Jo-1 (HisRS), PL7 (ThrRS), PL12 (AlaRS), EJ (GlyRS), KS (AsnRS), and Zo (PheRS). Only two Class I aaRS autoantibody targets, namely OJ (IleRS) and Ha (TyrRS) have been described so far [6]. Most IIM patients with anti-aaRS produce autoantibodies against a single aaRS, although in rare cases antibodies may occur directed to two aaRSs [36].

The anti-Jo-1 (or anti-HisRS) is the most frequently occurring autoantibody in PM/DM patients, its presence nearly excludes one of the three IIM, being inclusion-body myositis [37-39]. Different clinical and immunological features are associated with the presence of anti-Jo-1 antibodies, but whether the autoantibodies are a cause or a consequence of the development of the disease remains unknown, despite the fact that a correlation between the titer of anti-Jo-1 antibody and disease activity has been observed [40].

The major epitope of the Jo-1 molecule has been defined as a coiled-coil structure within the protein [41], although antibodies directed to other parts of the Jo-1 protein can be detected as well [42, 43]. Class switching, affinity maturation, species-specificity, and spectrotype broadening during the Jo-1 antibody response have been detected, which suggests a T cell-dependent and antigen-driven response in IIM patients [41, 43-45]. These processes also suggest that epitope spreading during the course of the disease may occur. Most studies that investigate the autoepitopes on the Jo-1 protein report activities that recognize multiple, both linear and conformation-dependent Jo-1 epitopes.

Myositis-specific autoantibody profiles are often associated with distinct clinical features [46]. Indeed, anti-aaRS antibodies are associated with a unique clinical syndrome, the so-called anti-synthetase syndrome. Characteristics of the anti-synthetase syndrome include myositis, Raynaud's phenomenon, arthralgia, fever, skin changes and interstitial lung disease, although clinical features may differ between patients with the anti-synthetase syndrome [47, 48]. The interstitial lung disease is important as it may negatively affect prognosis [49].

A number of studies investigated the genetic background of IIM patients and tried to correlate phenotypic characteristics with genetic predispositions. Besides associations with the human leukocyte antigen (HLA) 8.1 ancestral haplotype (HLA-DRB1*03 - DQA1*05 - DQB1*02), significant correlations were found between the presence of anti-Jo-1 antibody and HLA-DRB1*0301 and/or HLA-DPB1*0101 (reviewed by [50]). There are also data supporting the idea that candidate IIM autoantigens can be involved in the induction and propagation of the autoimmune response. The expression of the

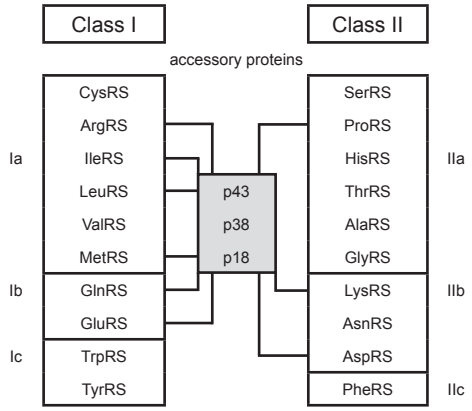

Fig. 6 Classification of aminoacyl-tRNA synthetases.
AaRSs are (sub)classified according to the chemical specificity of the reaction they catalyze and on the presence of conserved domains in their amino acid sequences (Class I and II, and subclasses a, b, and c). The aaRS that are associated with the multisynthetase complex (MSC) are connected to the three MSC accessory proteins (p43, p38, and p18) via bold lines.

Jo-1 antigen was found to be elevated in regenerating muscle of IIM patients compared to normal muscle [51, 52]. However, clear pathologic relevance of these antibodies in IIM has not been found yet.

How to assay for anti-aaRS antibodies
Commercially based LIAs can be used to test for several anti-aaRS antibodies, including anti-Jo-1, anti-PL7, and anti-PL12 see Fig. 4 and [13, 14, 53]. However, reaction conditions (e.g. temperature) have to be standardized, because they can have profound effects on the experimental outcome [13]. Recently, LIA strips have been developed that also detect anti-EJ and anti-OJ reactivities. Other less frequently found anti-aaRS reactivities such as anti-KS, anti-Ha, and anti-Zo are not commercially exploited yet. The latter anti-aaRSs are still being detected by the more conventional immunoblot and immunoprecipitation-based techniques.

Mi-2

Function
The chromatin in the nucleus of eukaryotic cells consists of a densely packed complex of DNA and proteins. The basic structure is the nucleosome, each of which contains an octamer of four core histones (H2A, H2B, H3, and H4) and a piece of approximately 150 basepairs DNA tightly wrapped around it. To allow gene transcription and chromosome replication, the chromatin is dynamically and orderly unfolded and, after replication or transcription repression, reformed. Chromatin structure remodeling is mainly regulated via two mechanisms reviewed by [54, 55]. The first involves covalent modification of nucleosomes such as methylation of DNA and (de-)acetylation of lysine residues in the core histones. The second mechanism involves dynamic changes of the histone-DNA interactions within the nucleosome in an ATP-dependent manner, which results in an increased or decreased accessibility of nucleosomal DNA (Fig. 7). The major remodeling enzymes involved can be categorized based on their ATPase subunits. These include the ISWI/SNF2L-type ATPases and the chromodomain helicase DNA-binding (CHD) protein family.
The Mi-2 protein was first identified as a DM-specific autoantigen and later shown to be a subunit of the nucleosome remodeling and deacetylation (NuRD) complex [56, 57]. The core subunits of the Mi-2/NuRD complexes are classified into the CHD protein family and are found to occur in different subunit compositions, depending on their functional activity reviewed by [58]. The components include histone de-acetylases (HDAC1 and HDAC2), retinoblastoma associated proteins (RbAp), metastasis-associated protein (MTA), a methyl-CpG-binding domain containing protein (MBD), and either one of the highly related Mi-2α (CHD3) and Mi-2β (CHD4) proteins [58-60]. The NuRD complexes are unique transcriptional regulators since they combine three enzymatic activities, namely histone deacetylation, histone demethylation, and ATP-dependent chromatin remodeling activities.

Potentially disease-related PTMs
The Mi-2/NuRD complex or Mi-2 protein complex has been described to be a substrate for modifying enzymes such as enzymes conjugating the small ubiquitin-related modifier (SUMO) and GrB [24, 61]. However, a direct relation between modified protein and IIM pathogenesis has not been demonstrated yet.

Antibodies to Mi-2 and their clinical associations
Reichlin and coworkers were the first to describe anti-Mi-2 antibodies in IIM patients and to date the anti-Mi-2 antibody can be detected in up to 20% of DM patients [62, 63]. The anti-Mi-2 positive patient sera are able to immunoprecipitate a major protein of approximately 240 kDa, together with up to 8 other components of the Mi-2 complex (200, 150, 72, 65, 63, 50, and 34 kDa) [56, 64](Fig. 1). Subsequently, two highly related Mi-2 proteins, Mi-2α and Mi-2β (calculated molecular masses are 220 and 218 kDa, respectively), which both contain epitopes that are recognized by anti-Mi-2 antibodies, have been identified as antigens in patients [57, 65]. Although anti-Mi-2 antibodies seem

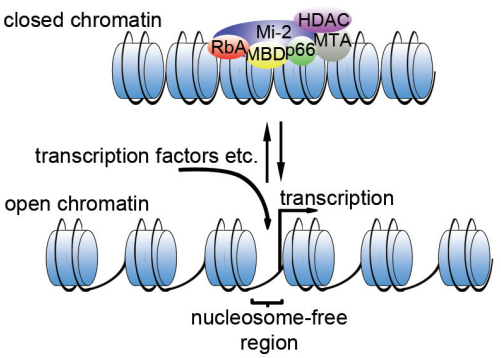

Fig. 7 Predicted model of chromatin remodeling by the Mi-2/NuRD complex.
The core subunits of the Mi-2/NuRD complex are suggested to be involved in modifying the chromatin structure, which can result in the initiation and/or maintenance of gene repression.

to be more tightly associated with DM, in several studies anti-Mi-2 positive PM and IBM patients have been detected as well [8, 66]. Therefore, cutaneous characteristics such as Gottron's papules, heliotrope rash, cuticular overgrowth and so-called V- and shawl sign, in combination with autoantibody profiles are considered to be the most reliable diagnostic features for DM [1]. Anti-Mi-2 positive patients tend to respond well to steroid therapy and thus have a relatively good prognostic profile [67].

Recent studies have found elevated Mi-2 expression levels in (regenerating) DM muscle biopsies and in certain tumors associated with autoimmune IIM [51, 68]. Additionally, Kashiwagi et al. described a crucial role of Mi-2 in the development and repair of the basal epidermis which may support a pathophysiological role for Mi-2 in the development of DM [69]. Moreover, latitudinal differences have been demonstrated to affect the occurrence of DM. These studies suggest an important role of environmental factors such as UV-light in the development of DM and anti-Mi-2 formation [70-73]. Anti-Mi-2 antibodies were detected in juvenile DM as well as adult DM, although autoantibody frequency may vary between the different DM subtypes [74-76]. Genetic predispositions in different HLA genes of (non-) Caucasians, but also protein tyrosine phosphatase and pro-inflammatory cytokine genes have been shown to be involved in the development of IIM reviewed by [50]. The HLA-DRB1*0302 and HLA-DRB1*0701 alleles have been described as risk factors for the generation of anti-Mi-2 antibodies in African-American and Caucasian individuals [50, 77].

How to assay for anti-Mi-2 antibodies

Several diagnostic assays have been used over the years to detect anti-Mi-2 antibody reactivities in patient sera. To date home-made tests are still more accurate, however, due to an enhanced reproducibility, sensitivity, and specificity, line-blot techniques will probably be preferred over the more conventional ELISA and IP assays in the future [53].

Signal Recognition Particle (SRP)

Function

Proteins that are involved in intercellular signaling are either being secreted or transported to the plasma membrane. The intracellular translocation of such proteins, from translational site to functional site, is regulated by a specialized secretory pathway. First, newly synthesized proteins have to be directed to the endoplasmic reticulum (ER), which is a signal peptide sequence driven process. This sequence is located at the N-terminus of the protein, and protein translocation can occur post-translationally or co-translationally. Co-translational translocation involves the so-called signal recognition particle (SRP), which is a ribonucleoprotein (RNP) complex that consists of six proteins (9, 14, 19, 54, 68, and 72 kDa) and a RNA molecule of approximately 300 nucleotides, termed 7SL RNA [78]. In the SRP complex two distinct RNP domains can be distinguished (Fig. 8) [79-81]. The S-domain consists of SRP19, SRP54, SRP68, SRP72, and a forked 7SL RNA moiety. It recognizes the signal peptide on the growing peptide chain and targets the complex to the ER membrane. The Alu-domain is formed by SRP9, SRP14 at the opposite end of the 7SL RNA and probably causes a transient translational arrest until the ribosome/nascent chain complex has docked to the ER. The SRP particle has a high affinity for ribosomes containing a protruding signal peptide. After binding, the SRP-ribosome complex binds the SRP receptor (SR) on the ER membrane via GTP-dependent interactions. Subsequently, the ribosome can dock onto the translocon and the binding between signal sequence and SRP complex is broken, allowing translation to resume. Finally, after GTP hydrolysis, SRP and the SR dissociate and the SRP complex is recycled for a following round of translocation reviewed by [82].

Potentially disease-related PTMs

Casciola-Rosen *et al.* described SRP72 as a substrate for GrB and suggested a pathogenic role for the unique fragments generated in this way [24].

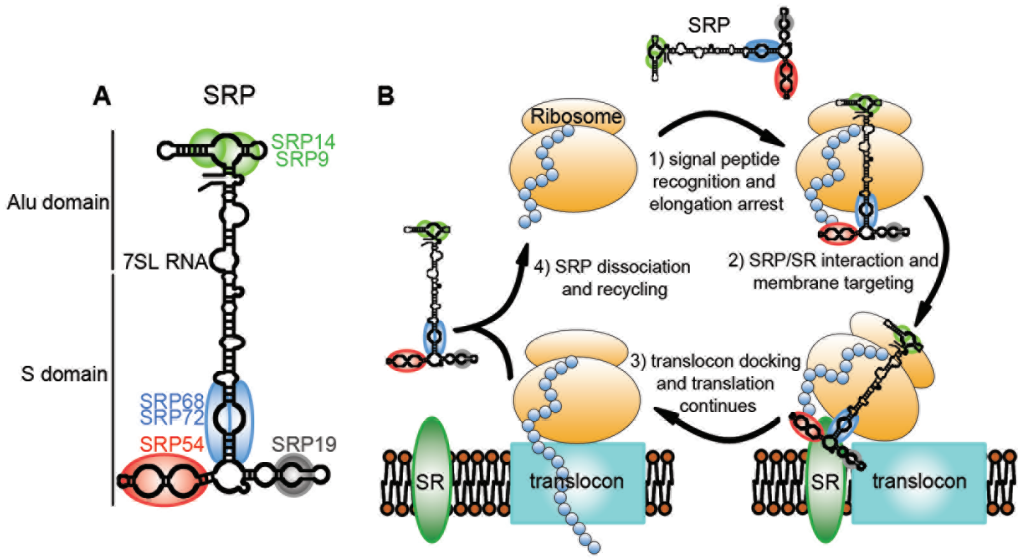

Fig. 8 SRP mediates translocation of nascent, signal peptide-containing proteins.
(**A**) Schematic structure of SRP. (**B**) Mechanism of protein translocation mediated by SRP. First, SRP binds to the N-terminal signal peptide of the growing peptide chain which results in elongation arrest (1). Subsequently, the ribosome/SRP complex is targeted to the SRP receptor (SR) at the ER membrane by specific sequences in the SRP proteins (2). Docking of the complex to the membrane bound translocon releases the signal peptide from the SRP complex and enables translation to continue (3). The SRP dissociates and is recycled for additional translocation events (4).

Antibodies to SRP and their clinical associations

Reeves and colleagues were the first to describe the SRP as an autoantigen in IIM patients [83]. Autoantibodies directed to SRP54 are most frequently found, although anti-SRP72, anti-SRP68, and anti-7SL RNA reactivities have been reported as well [84-86]. Patients that generate anti-SRP antibodies (up to 5% of patients with IIM) appear to form a distinct clinical and histopathological subset within the IIM [84, 87]. The so-called necrotizing myopathy seen in anti-SRP positive patients, include necrotic and regenerating myofibers. Muscle enzyme elevations as well as lower numbers of infiltrating lymphocytes are found in muscle biopsies compared to PM. In addition, patients with anti-SRP antibodies frequently reveal an unusually severe weakness, acute onset, rapid disease progression, and resistance to treatment [84, 87, 88]. These distinct characteristics emphasize that anti-SRP antibodies can be used as a biomarker for a distinct subgroup of IIM patients. Anti-SRP antibodies are rarely detected in juvenile IIM [6].

How to assay for anti-SRP antibodies

Anti-SRP antibodies, similar to anti-aaRS, anti-Mi-2, and several anti-MAA, can be detected via commercial LIAs. However, Ronnelid and colleagues found discrepancies in the autoantibody reactivities when commercial LIAs were used at different temperatures. This temperature sensitivity is most pronounced for weakly positive and negative samples, which may result in false positive or false negative diagnoses [13]. This emphasizes the importance of further evaluation and development of commercial LIAs, in order to ensure that specificity and sensitivity are warranted.

CADM-140

Function

The innate immune system utilizes a specific group of receptors, the so-called pattern recognition receptors (PRRs), to detect specific pathogenic components [89]. Three major classes of PRRs have been identified, including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and nucleotide-oligomerization domain (NOD)-like receptors (NLRs). The melanoma differentiation-associated gene 5 (MDA5), or CADM-140 autoantigen, together with RIG-I and LGP2 (laboratory of genetics and physiology 2) are members of the RLH helicase family. Both RIG-I and MDA5 contain N-terminal caspase-recruitment domains (CARDs) followed by a DExD/H box RNA helicase domain, whereas LGP2 has a similar helicase domain but lacks a CARD domain. LGP2 has been suggested to function as a negative regulator of RIG-I/MDA5 signaling [90-92]. The TLRs and RLHs are able to sense viral RNA and DNA components and downstream signaling results

in the activation of cells to produce type I interferons (IFNs) and pro-inflammatory cytokines. These factors stimulate the immune system by activating natural killer (NK) and T cells, and promote the induction of apoptosis of virus-infected cells and the cellular anti-viral response that interferes with cellular and viral processes.

Potentially disease-related PTMs

Barral and colleagues describe MDA5/CADM-140 degradation products, both in puromycin-induced apoptotic HeLa cells and poliovirus-infected cells, which is suggested to antagonize the type I IFN-induced anti-viral response [93]. Disease-associated MDA5/CADM-140 modifications have not been described yet.

Antibodies to CADM-140 and their clinical associations

The IIM classification criteria enable clinicians to categorize several distinct IIM populations. Some individuals, the so-called amyopathic DM patients, exert typical skin manifestations of DM but little or no evidence of clinical IIM. When patients do not develop clinical symptoms of IIM for at least 2 years after the onset of skin manifestations they will be classified as clinically-amyopathic DM (CADM) [94]. Japanese patients with CADM develop a rapidly progressive interstitial lung disease (ILD), which frequently appears to be resistant to treatment and which may lead to early death [95, 96]. Sato and colleagues were the first to describe a novel antibody (anti-CADM-140) that immunoprecipitated a 140-kDa protein in 53% of the CADM patients, whereas no such antibodies were detected in any of the PM or DM patients [97] (Fig. 1). Recently, it was shown that this 140-kDa protein is identical to MDA5 [98]. It is intriguing that anti-CADM-140 antibodies are only reported to occur in Japanese patients so far. Nakashima and colleagues confirmed the anti-CADM-140 antibody to be specific for CADM, although a few positive patients showed symptoms of IIM (DM according to criteria) [99]. A recent report from Hoshino *et al.*, suggested that anti-CADM140/MDA5 autoantibodies could be used as a novel serological marker to distinguish between different DM subsets and associated clinical complications [100].

How to assay for anti-CADM-140 antibodies

No commercial assay is available yet for the detection of the anti-CADM-140 antibody. Sato and colleagues used immunoprecipitation, immunoblotting and indirect immunofluorescence techniques to detect this specificity [97]. In addition, an ELISA, containing the recombinant CADM-140 protein as antigen, was used to test for anti-CADM-140 antibodies with a fairly high sensitivity and specificity [98]. The recombinant CADM-140 antigen might be suitable for the development of a commercial test.

p155/140

Function

The Tif1-γ, or p155/140 protein has been characterized as a member of the transcriptional intermediary factor 1 (Tif1) gene family [101-103]. Similar to the other Tif1 members, Tif1-α and Tif1-β, Tif1-γ contains several specific protein domains, including a RING finger, B-boxes, a coiled-coil domain, a PHD/TTC domain, and a bromodomain involved in protein-protein interactions. Several possible functions of the Tif1-γ protein have been described, including regulation of transcription and maintenance of tissue homeostasis [104, 105].

While the antigenic p155 protein (one of the two antigenic proteins) has been identified as Tif1-γ, the identity of the p140 protein remains elusive [103].

Potentially disease-related PTMs

No modifications have been associated with disease.

Antibodies to p155/140 and their clinical associations

Several studies indicated that anti-p155/140 antibodies appear to be associated with adult and juvenile DM (JDM) patients [106]. Up to 23% of DM patients seem to contain autoantibodies reactive with a 155/140-kDa protein doublet (Fig. 1). The positive adult DM patients often have more severe cutaneous involvement and an increased risk for malignancies (particularly adenocarcinomas) [106-109]. Follow-up studies confirmed the cancer-associated DM (C-ADM) subset specificity of the anti-p155/140 antibody [100]. The anti-p155/140 antibody is detected in JDM sera with a similar frequency as in adult DM, although the association with malignancy in JDM was not observed [110].

How to assay for anti-p155/140 antibodies

Anti-p155/140 was first identified using an immunoprecipitation procedure with C-ADM patient sera and radiolabeled K562 cell extracts and immunoblotting [106]. Recently, the immunoprecipitation of biotinylated recombinant Tif1-γ was used to evaluate the clinical features associated with anti-p155/140 in DM patients [100]. The availability of such recombinant proteins might encourage the development of a commercial diagnostic test.

Small ubiquitin-like modifier activating enzyme (SAE)

Function

Many enzymes are involved in post-translational protein modifications, and thereby regulate protein activity, subcellular localization, stability and protein-protein interactions. These protein modifications can be a response to environmental triggers or depend on the cellular state. The SUMO protein family is structurally related to ubiquitin, and, like ubiquitin, can be post-translationally conjugated to other proteins by a similar mechanism. SUMO needs to be activated before post-translational conjugation can occur. This process is regulated by the SUMO-activating enzyme (E1) and involves the small ubiquitin-like modifier activating enzyme (SAE), which consists of a heterodimer of SAE1 and SAE2. Sumoylation is found to be involved in a variety of processes such as nuclear protein transport, DNA replication and repair, and cell division [111]. In addition, the number of substrates is increasing and emphasizes the diverse roles of SUMO modification in many cellular processes [112]. Although several SUMO family members have been shown to play role in the sumoylation of proteins, information about the consequences of sumoylation on the functions of these proteins is rather scarce.

Potentially disease-related PTMs

No disease-associated modifications have been documented.

Antibodies to SAE and their clinical associations

Betteridge and colleagues reported a novel autoantibody in adult DM patients that immunoprecipitated two uncharacterized bands at 40 and 90 kDa [113]. Further proteomic analysis identified the autoantigenic targets as small ubiquitin-like modifier activating enzyme (SAE) subunits. A large scale autoantibody screening by IP identified anti-SAE antibodies in 8.4% of DM patients. These results suggest that anti-SAE may identify a new serological IIM subset that present initially CADM and progress to develop IIM with a high frequency of systemic features except interstitial pneumonia.

How to assay for anti-SAE antibodies

Anti-SAE antibodies can be detected by immunoprecipitation and show a diffuse, coarse, speckled, nucleolar-sparing pattern in indirect immunofluorescence analysis [113, 114].

p140

Function

The NXP-2 protein is a recently identified nuclear matrix associated protein. Ectopically expressed NXP-2 mutants and structure prediction programs allowed the identification of several functional domains for NXP-2, including RNA-binding, nuclear matrix-binding, and coiled-coil domains [115]. Northern blot analysis showed NXP-2 to be expressed in specific tissues and at different levels. Moreover, RNA-binding and nuclear-binding capacities of NXP-2 suggest a similar role in the control of posttranscriptional processes as are described for other nuclear matrix proteins that contain similar domains [116]. Rosendorff and coworkers reported a transcriptional repressor activity for the NXP-2 *in vitro*, which may be mediated by SUMO modification [117]. These results suggest that NXP-2 may be involved in tissue-specific RNA metabolism and maintenance of nuclear structure rather than being a constitutive factor in nuclear functions.

Potentially disease-related PTMs

NXP-2 was recently described to be a candidate substrate for SUMO modification, which might result in the down-regulation of transcription [112, 117]. However, whether this results in the initiation of pathogenic mechanisms leading to immunological dysregulation is not known yet.

Antibodies to p140 and their clinical associations

Oddis and colleagues described autoantibodies directed against a 140-kDa target (anti-MJ) in a cohort of juvenile IIM patients [118]. Later, Gunawardena and co-workers identified antibodies to a 140-kDa protein in 23% of juvenile IIM sera that form a distinct JDM subset [119]. The p140 autoantigen targeted by these antibodies is different from the p155/140 antigen described above. Immunodepletion experiments with reference anti-p140-positive sera and anti-NXP-2 monoclonal antibodies suggested that the p140 protein is identical to the MJ autoantigen. Recent studies of Targoff's group characterized the p140/MJ antigen and described the p140 protein as the nuclear matrix protein (NXP-2) [120].

The presence of this anti-p140 antibody is associated with calcinosis, which defines a different clinical phenotypic JDM subset.

How to assay for anti-p140 antibodies

Radiolabeled immunoprecipitation assays have been used to identify the presence of this antibody activity [119].

Novel MSAs

Christopher-Stine and colleagues recently described a novel autoantibody in a subset of individuals suffering from necrotizing myopathy, which previously were considered to be autoantibody negative [121]. They studied 255 patients with myopathies and found 38 muscle biopsies with necrotizing myopathies. Twenty-six patients were diagnosed as autoantibody negative, whereas in the remaining 12 sera anti-SRP or anti-aaRS activities were detected. Screening of the 26 negative sera for putative novel autoantibody reactivities by immunoprecipitation resulted in the detection of two antigenic proteins with molecular weights of approximately 200 kDa and 100 kDa in 62% of these patients. The evaluation of clinical data suggested that patients with anti-200/100 antibodies form a separate group of necrotizing myopathies that are associated with the exposure to statin medication and are responsive to immunosuppression. Further characterization of the anti-200/100 target might contribute to the understanding how this particular subset can develop.

Concluding remarks and perspectives

The characterization of MSA and the development of methods to detect these autoantibodies in patient sera for diagnostic purposes represent an active field of research. A series of novel MSA have been identified during the last decade and new technologies have been developed for their identification and detection, especially using multiplex assays. The application of multiplex assays will not only facilitate the simultaneous detection of multiple autoantibody reactivities, MSA as well as MAA, but may also allow the identification of autoantibody profiles that might have additional diagnostic or prognostic value.

Although the prevalence of MSAs in IIM patients is generally relatively low (< 20%), for a number of them clinical associations have indeed been identified. Several reports indicate that MSAs are able to discriminate between distinct disease entities and/or can be correlated to disease severity, which suggests that MSAs might help define IIM subtypes and predict disease progression, and/or treatment. The number of MSA is steadily growing and the diverse functions and subcellular localizations of the associated autoantigenic targets suggest selective mechanisms in the development of these autoantibodies. Whether autoantibodies directly contribute to the development and/or progression of IIM, however, remains elusive. All MSA that have been characterized up to now are associated with PM and/or DM. An intriguing question is whether MSA are also produced by patients with the third major type of IIM, IBM.

The detection of novel autoantibody targets in the different immune-mediated myopathies emphasizes the complexity of the mechanisms that define the development of these autoimmune diseases. Further studies that elucidate structure and function of autoantigenic targets will be essential in understanding the underlying pathological mechanisms in IIM.

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Chapter 3

Modification of autoantigenic proteins in oxidatively stressed Jurkat cells

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Abstract

Autoimmune diseases are believed to develop due to a loss of immunological tolerance, which may result in the formation of autoantibodies and/or the destruction of tissue. Post-translational modifications of autoantigens have been proposed to play a role in the generation of autoantibodies. In addition to apoptosis- or necrosis-induced modifications, oxidative stress-induced modifications are also believed to play a role in the generation of such neo-epitopes. To investigate whether oxidative stress leads to the modification of autoantigenic proteins in SLE, PM/Scl overlap syndrome and myositis, we analyzed these proteins from oxidatively stressed Jurkat cells as well as their purified recombinant equivalents by SDS-PAGE and immunoblotting. A time and H_2O_2-concentration dependent retarded electrophoretic mobility was observed for all analyzed proteins. *In vitro* oxidized recombinant U1A and Jo-1 proteins resulted in mobility shifts that were comparable with those observed with H_2O_2-treated Jurkat cells. The retarded migration of U1A, Rrp40, Rrp41, Ro60, La, and Jo-1 in SDS-PAGE can result from the oxidative modification of these proteins upon H_2O_2 treatment. Hydroxyl radicals are suggested to be involved in the oxidative modification, because we observed similar results with hypochlorite treated Jurkat cells and recombinant proteins. These modifications may result in the increased autoantigenicity of the protein to which the immune response in the respective autoimmune diseases is directed.

Introduction

Neo-epitopes generated by modification of self-proteins may trigger the loss of immunological tolerance in autoimmune diseases. Since this hypothesis was formulated, researchers have focused on finding processes which may cause such alterations in self-proteins. Cell death is a process often accompanied by post-translational modifications. Both apoptotic and necrotic modifications have been reported to occur in a variety of autoantigens [1,2]. Reactive oxygen species (ROS)-mediated reactions, which can be associated with necrosis, may also be of importance for the modification of self-proteins. One of the mechanisms that are used by the innate immune system to destroy foreign pathogens is the rapid release of ROS, a process which is called oxygen- or respiratory burst. ROS are oxygen-based molecules which are highly reactive and include superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). ROS can be formed intracellularly, in for example the mitochondria, as a natural byproduct of oxygen metabolism. Under stress conditions ROS levels can increase dramatically and damage cellular structures, including DNA, proteins and lipids [3-5]. Cell damage often results in apoptosis or necrosis.

Antigens in a variety of autoimmune diseases, including systemic lupus erythematosus (SLE), type 1 diabetes (T1D), and rheumatoid arthritis (RA) have been shown to be oxidatively modified [6,7]. The observed oxidative modifications of the antigens targeted in these diseases can be diverse. For example, Ro60, a 60 kDa antigenic target for antibodies present in a subset of SLE patients, can be modified by lipid oxidation products resulting in the formation of aldehydes, mainly 4-hydroxy-2-alkenals. In addition, during inflammation non-enzymatic reactions can result in the production of so-called “advanced glycation end” products (AGE), which can be associated with proteins [8]. AGE-modified antibodies are found amongst others in rheumatoid arthritis patients and are believed to be the result of inflammation and/or oxidative stress [9]. The generation of anti-IgG-AGE appears to be specific for a subset of RA patients suggesting that the modification of distinct proteins can result in different clinical phenotypes [10].

To further investigate whether oxidative stress conditions are able to cause distinct post-translational modifications (PTM) of autoantigenic proteins, we studied the effects of ROS-inducing compounds on intracellular proteins.

Materials and Methods

Cell lines

Jurkat (human T cell lymphoma) cells were cultured in RPMI 1640 GlutaMAX™I (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL), 25 mM Hepes-KOH, pH 8.0, 1 mM penicillin, 1 mM streptomycin, and 1 mM sodium pyruvate. The cells were grown in a humidified 37 ºC incubator containing 5% CO_2 and maintained at a cell density of 0.5 to 1.2 x 10^6 cells/ml.

Induction of oxidative stress and cell extract preparation

Oxidative stress was generally induced by incubation of Jurkat cells in culture medium supplemented with 48 mM H_2O_2 (Acros Organics; Geel, Belgium) for 5 hours, or as indicated. Alternatively, Jurkat cells were subjected for the indicated periods of time to 15 mM NaOCl in culture medium. Cells were harvested by centrifugation and resuspended in sonication buffer (20 mM Hepes-KOH, pH 7.4, 100 mM KCl, 0.5 mM PMSF, 0.1% NP-40, 2 mM EDTA, 1 mM DTE) at a concentration of 10^8 cells/ml and lysed by sonication on ice. Cell debris was removed by centrifugation for 15 min at 16,000xg and supernatants were stored at -20 °C until use.

In vitro oxidative modification

Recombinant U1A and Jo-1 proteins were purified as described below, after which the buffer was exchanged with KRB-buffer (20 mM Hepes-KOH, pH 7.4, 127 mM NaCl, 5.5 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, and 2 mM $MgSO_4$) using over night dialysis at 4 °C in 10 kDa cut-off Spectra/Por® 6 tubes (VWR). Recombinant proteins were subjected to oxidative conditions generated by the Fenton reaction by incubation with 100 µM $Fe(SO_4)$ and 1.7 mM ascorbic acid in KRB-buffer with or without 13 mM H_2O_2 for 30 minutes. The reaction was stopped by adding 10 mM EDTA. Alternatively, recombinant U1A and Jo-1 protein was subjected for up to 30 minutes to 1.5 mM NaOCl in KRB-buffer.

Gel electrophoresis and immunoblotting

Extracts from normal or oxidized Jurkat cells and material from mock or in vitro oxidized recombinant proteins were separated by electrophoresis in polyacrylamide gels and stained with colloidal Coomassie Brilliant Blue (CBB; 0.08% CBB G250, 1.6% ortho-phosphoric acid, 8% ammonium sulfate and 20% methanol) or transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with 5% non-fat dried milk in PBS containing

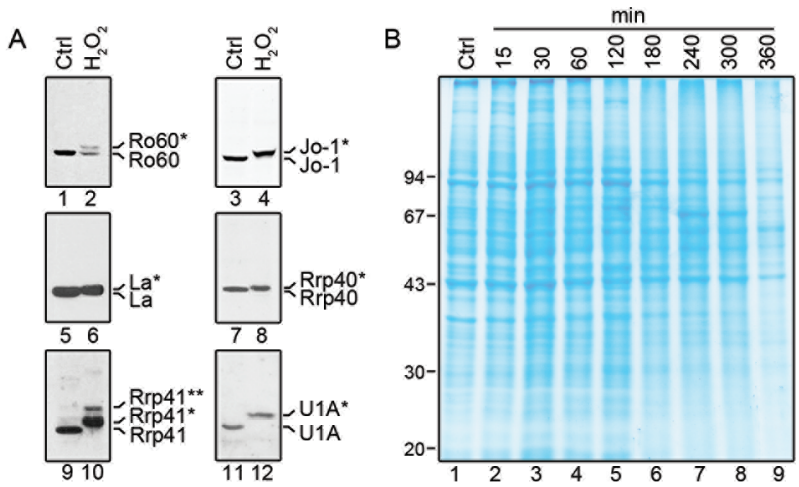

Fig. 1 Hydrogen peroxide-induced autoantigen modification in Jurkat cells.
(**A**) Extracts of Jurkat cells which were treated for 5 hours with 0.15% H_2O_2 (H_2O_2) or extracts of non-treated Jurkat cells (Ctrl) were analyzed by immunoblotting. Western blots containing the respective extracts were incubated with patient sera containing antibody reactivities against U1A or Jo-1, mouse monoclonal antibodies reactive with Ro60 and La or with rabbit antibodies directed to the exosome proteins Rrp40 and Rrp41. The positions of the modified proteins are indicated with asterisks. (**B**) Jurkat cells were cultured for 0 – 360 min after the addition of H_2O_2 to a final concentration of 0.15%. Cell extracts were separated by SDS-PAGE and stained with Coomassie Brilliant Blue.

0.05% Tween-20 (MPBST). Mouse monoclonal antibodies (anti-U1A, 9A9, anti-Ro60, 1D11, and anti-La, SW5), rabbit polyclonal antibodies (anti-Rrp40, H70, anti-Rrp41, H71) and human patient sera (anti-Jo-1, anti-U1-70k and anti-topoisomerase I) were diluted in MPBST prior to the detection of proteins on western blot. As secondary antibody, horseradish peroxidase-conjugated rabbit-anti mouse IgG, swine anti-rabbit IgG or rabbit-anti human IgG (Dako Immunoglobulins) were used, respectively, in MPBST. Bound antibodies were visualized by chemiluminescence.

Recombinant protein production and purification
The recombinant U1A and Jo-1 proteins were expressed in *E. coli* and purified as described previously [11,12]. The purified proteins were pooled and stored at -80 °C until use.

Results

Autoantigens are modified in oxidatively stressed Jurkat cells
Reactive oxygen species such as $OH^{\bullet}$, $O_2^{\bullet -}$, and H_2O_2 are able to induce cell death. Saito and colleagues describe apoptotic and necrotic phenotypes in Jurkat cells that were exposed to low or high concentrations of H_2O_2, respectively [13]. The radicals generated during oxidative stress may lead to distinct cleavage products (Hof *et al.* unpublished results) or oxidation specific modifications in biomolecules that trigger an immune response. Jurkat cells were subjected to similar levels of oxidative stress as described by Casiano *et al.*, by adding 48 mM H_2O_2 to the medium followed by culturing for 5 hours [1]. Cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting. For all autoantigenic proteins analyzed (Ro60, La, Jo-1, U1A) a retarded electrophoretic mobility was observed (Fig. 1A). In addition, a mobility shift was observed for two human exosome core proteins, Rrp40 and Rrp41 (lanes 8, 10). Interestingly, in the case of Rrp41 two retarded species were observed. For the Ro60 antigen and possibly the La protein, not all molecules appeared to be modified after 5 hours in the presence of H_2O_2, since a signal similar to the non-modified sample could be detected as well (lanes 2, 6). Because the migration of all proteins analyzed by immunoblotting appeared to be affected, cells were subjected to H_2O_2-induced oxidative stress for various periods of time and the migration of all proteins detectable by total protein staining of the SDS-PAGE gels was examined. A highly similar staining pattern was observed up to 180 min, after which several changes became apparent (Fig. 1B). These data indicate that after several hours of oxidative stress many proteins undergo changes that affect their electrophoretic mobility in SDS-PAGE.
To investigate the rate of modification of the autoantigenic proteins, their electrophoretic mobility was determined after different incubation times with 48 mM H_2O_2. The results revealed strong differences

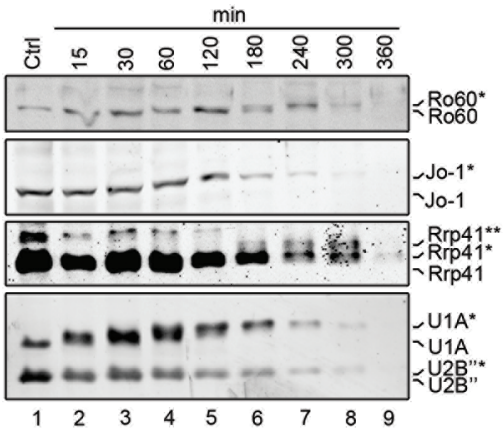

Fig. 2 Kinetics of autoantigen modification in oxidatively stressed Jurkat cells.
Jurkat cells were treated with 0.15% H_2O_2 for the indicated periods of time. Cell extracts were separated by SDS-PAGE and specific proteins were visualized by immunoblotting using a patient serum containing antibodies directed against Jo-1, mouse monoclonal antibodies to the U1A and Ro60 proteins, and a rabbit polyclonal antibody against Rrp41. The anti-U1A monoclonal antibody cross-reacts with the U2B'' protein. Modified proteins are indicated with asterisks.

between the rates by which the different proteins were modified. At 180 minutes the Ro60 antigen shows a transition of the non-modified to the oxidized protein (Fig. 2), and after 240 minutes the conversion is virtually complete. The Jo-1 and U1A proteins were completely modified after 120 and 180 min, respectively. Rrp41 was shifted completely after 120 min, whereas the second retarded Rrp41 species appeared only after 180 min. The monoclonal antibody that was used for the detection of U1A is cross-reactive with the U2B'' protein [14]. Also U2B" appeared to be shifted, which was already detectable after 15 minutes and became complete after 60 min. It should be noted that the signal intensities obtained with material from cells exposed for more than 180 minutes to H_2O_2 tended to decrease, which may result from the loss of membrane integrity and intracellular content or from the disruption of the epitopes recognized by the antibodies used (Fig. 2).

Concentration-dependent modification of autoantigens

To investigate whether similar oxidative modifications were observed at milder conditions, Jurkat cells were treated with different concentrations of H_2O_2, ranging from 0.03 mM to 48 mM, for 5 hours. Only at the higher concentrations, 32 mM and 48 mM H_2O_2, a mobility shift for the U1A protein was observed (Fig. 3). The migration of U2B" was also affected at the higher H_2O_2 concentrations, although this did not seem to be as pronounced as that of U1A. The relatively high

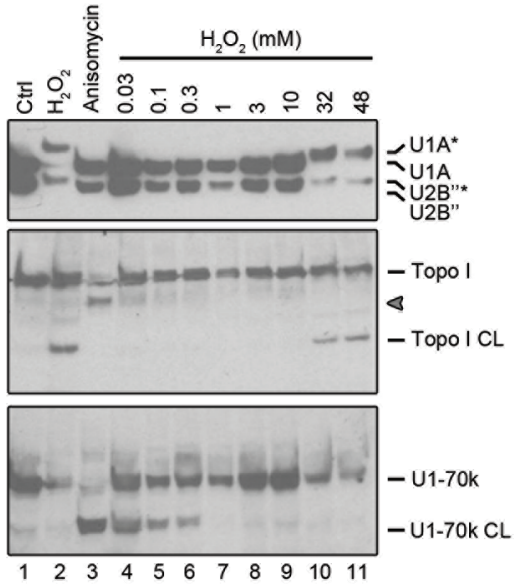

Fig. 3 Hydrogen peroxide concentration-dependent autoantigen modification.
Jurkat cells were cultured in the presence of various concentrations of H_2O_2 for 5 hours, after which cell extracts were analyzed by immunoblotting (lanes 4-11). Western blots containing extracts of untreated (Ctrl) and treated cells were incubated with patient sera containing autoantibodies against DNA topoisomerase I (Topo I) or U1-70k. U1A was detected with a monoclonal antibody that cross-reacts with the U2B'' protein (top panel). The modified protein signals are indicated with asterisks. As additional controls, cells were treated with 0.15% H_2O_2 for 5 hours to induce necrosis (lane 2), or 8 hours with 40 μM anisomycin to induce apoptosis (lane 3). The induction of necrosis was monitored by the appearance of the 45 kDa cleavage product of Topo I, Topo I CL (middle panel), whereas the induction of apoptosis was monitored by the appearance of the 40 kDa cleavage product of U1-70k, U1-70k CL (bottom panel). Note that Topo I in apoptotic cells is cleaved into a 70 kDa product (arrowhead), in agreement with previous observations.

concentrations of H_2O_2 may induce cell death and therefore the migration alterations observed may be associated with cell death. Hydrogen peroxide has been reported to induce necrosis [1], and therefore the induction of necrosis was monitored by the cleavage of topoisomerase I (Topo I), which leads to a characteristic 45 kDa cleavage product. Indeed, the Jurkat cells appeared to become necrotic after exposure to the H_2O_2 concentrations that led to U1A and U2B" modification (Fig. 3) [1]. In contrast, relatively low H_2O_2-concentrations (0.03 mM – 0.3 mM) induced apoptosis, as demonstrated by the specific fragmentation of U1-70 kDa (Fig. 3). These concentrations did not result in a detectable mobility shift of the U1A and U2B'' proteins. Jurkat cells rendered apoptotic by anisomycin treatment were analyzed in parallel as a reference. Note that, as reported previously [1], also the Topo I protein was cleaved in apoptotic cells, resulting in a cleavage

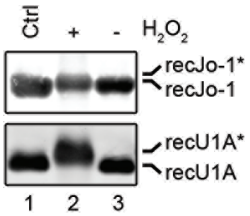

Fig. 4 *In vitro* oxidation of recombinant Jo-1 and U1A.
Recombinant Jo-1 (recJo-1) and U1A (recU1A) proteins (Ctrl) were incubated under oxidative conditions generated by the Fenton reaction. Material from reactions supplemented with H_2O_2 is indicated with + (lane 2), whereas material from reactions without H_2O_2 is labeled - (lane 3). Proteins were visualized using a patient serum containing anti-Jo-1 or a monoclonal anti-U1A antibody. The positions of the retarded proteins (lane 2) are indicated with asterisks.

product that is different from that in necrotic cells. These data suggest that the observed oxidative modification of antigens is primarily associated with H_2O_2 concentrations that induce necrosis.

In vitro oxidation of purified recombinant autoantigenic proteins

The exposure of Jurkat cells to H_2O_2 results in the elevation of both extracellular and intracellular ROS levels. To investigate whether hydroxyl radicals, which are the main direct ROS emerging from H_2O_2, are involved in the modification reactions, purified recombinant autoantigenic proteins were exposed to hydroxyl radicals generated by the Fenton reaction [15-18]. In this reaction ferrous iron (Fe^{2+}) is oxidized by H_2O_2 to yield ferric iron (Fe^{3+}), a hydroxyl anion (OH^-) and a hydroxyl radical ($OH^{\cdot}$). Both U1A and Jo-1 were oxidized *in vitro*, after which the proteins were analyzed by immunoblotting using a mouse monoclonal anti-U1A antibody and a anti-Jo-1 positive patient serum. The recombinant proteins were incubated under conditions generated by the Fenton reaction either in the presence of or the absence of H_2O_2 (Fig. 4). After incubation in the presence of H_2O_2 the migration of both U1A and Jo-1 in SDS-PAGE appeared to be retarded, very similar to what was observed for these proteins from H_2O_2 treated Jurkat cells. The mobility shift was not observed when the proteins were incubated without H_2O_2. The results suggest that the observed protein modifications are a direct effect of H_2O_2 or its products generated in the Fenton reaction.

Oxidative stress-induced protein modification is $O_2^{\cdot-}$ independent

To obtain more insight into the ROS species that are responsible for the detected modifications in Jurkat cells, we incubated these cells with two other ROS generators, menadione and paraquat, which

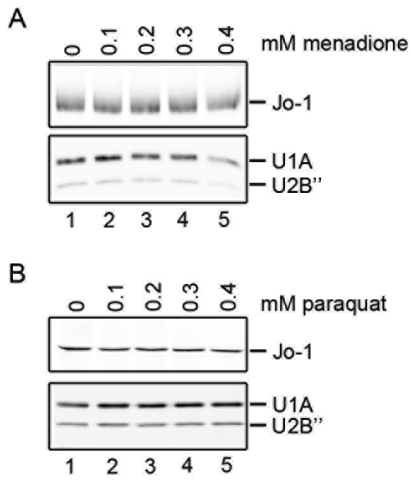

Fig. 5 The effect of superoxide-induced oxidative stress on autoantigenic proteins.
Jurkat cells were treated for 2 hours with various concentrations of menadione (**A**) or 24 hours with various concentrations of paraquat (**B**). Cell extracts were separated by SDS-PAGE and analyzed by immunoblotting using an anti-Jo-1 positive patient serum or a monoclonal anti-U1A/anti-U2B" antibody.

generate $O_2^{\cdot-}$ inside the cell [19,20]. Jurkat cells treated with up to 0.4 mM menadione (Fig. 5A) or up to 0.4 mM paraquat (Fig. 5B) were lysed and analyzed by immunoblotting and FACS analysis. After treatment intracellular superoxide anion levels were monitored in Jurkat cells using FACS analysis and the fluorescent hydroethidine probe (data not shown). Although both menadione and paraquat treatment did result in an increase in the intracellular superoxide anion concentration, neither menadione nor paraquat led to the detectable modification of the U1A and Jo-1 proteins.

Hypochlorous acid-induced autoantigen modification

Since H_2O_2 may lead to the production of $OH^{\cdot}$ in cells, it is possible that H_2O_2-derived $OH^{\cdot}$ plays a role in the observed modification of proteins. Neutrophils and monocytes express the enzyme myeloperoxidase (MPO), which plays a pivotal role in the destruction of pathogens. MPO produces hypochlorous acid (HOCl), which can react with many biomolecules, e.g. oxidize thiols, damage side-chains, or induce the fragmentation or aggregation of proteins [21]. However, through its reaction with $O_2^{\cdot-}$ or Fe^{2+}, HOCl also is a potent generator of the highly reactive $OH^{\cdot}$ radical [22]. To investigate whether $OH^{\cdot}$ is involved in electrophoretic mobility shifts of proteins as have been observed after exposure to H_2O_2, Jurkat cells or recombinant proteins were subjected to the alternative $OH^{\cdot}$ generator, HOCl. Subsequent to

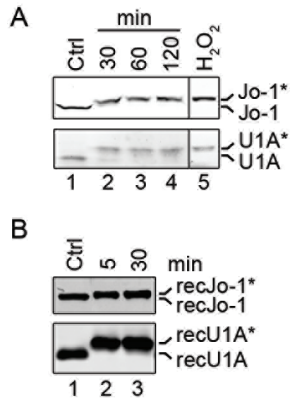

Fig. 6 The effect of hypochlorous acid on Jo-1 and U1A.
(**A**) Jurkat cells were treated with 0.1% NaOCl for the indicated periods of time (minutes; lanes 2-4), after which cell extracts were prepared and analyzed by immunoblotting using monospecific anti-Jo-1 or anti-U1A antibodies. In lane 1 material from untreated cells was loaded (Ctrl). Lane 5 contains extract of Jurkat cells that were treated for 2 hours with 0.15% H_2O_2. The positions of modified proteins are marked with asterisks. (**B**) Recombinant Jo-1 (recJo-1) and U1A (recU1A) proteins were treated either for 5 or 30 min with 0.01% NaOCl and visualized by immunoblotting as in panel A. The positions of recJo-1 and recU1A in treated samples are indicated with asterisks.

0.1% or 0.01% NaOCl treatment of Jurkat cells or recombinant proteins, respectively, samples were analyzed via immunoblotting. Both Jo-1 and U1A proteins from Jurkat cells showed a mobility shift already 30 min after the addition of NaOCl, which resulted in a similar electrophoretic mobility as observed after H_2O_2 exposure (Fig. 6A). RecJo-1 and recU1A became modified upon NaOCl treatment as well (Fig. 6B). A migration shift that was comparable to that upon H_2O_2, exposure was already detected after a 5 minute incubation in the presence of NaOCl. Since mobility shifts in NaOCl treated samples were highly similar to those observed under H_2O_2 conditions and the chemical nature of radicals after H_2O_2 and NaOCl decomposition resemble each other, suggest that $OH^{\cdot}$ radicals are responsible for the observed electrophoretic differences.

Discussion

The post-translational modification of proteins can generate neo-epitopes, which in predisposed individuals can initiate an immune response and eventually lead to the loss of immunological tolerance and autoimmunity. Here we report that autoantigenic proteins associated with various autoimmune diseases are modified upon their exposure to $OH^{\cdot}$. Oxidative modifications can have a variety of effects on the affected biomolecule, e.g. loss or gain of function. In rabbits that have been immunized with oxidized Ro60 an accelerated disease process was reported, in contrast to their non-oxidized counterparts [23]. A similar mechanism might be involved in the loss of immunological tolerance in autoimmune patients.

The altered electrophoretic mobility of Jo-1 is in agreement with the results of our previous studies on the oxidative modification of Jo-1 in H_2O_2-treated Jurkat cells (Van Dooren *et al.,* manuscript submitted for publication). The effects of H_2O_2 treatment were less pronounced for the Rrp40 and La proteins, which may be explained by differences in the number or microenvironment of amino acids that can be oxidized. In addition, oxidative modification of one residue could result in a conformational change of the protein and a concomitant increased susceptibility to oxidation of other amino acids. This could explain the step-wise modification as observed for Rrp41.

The total protein analysis of the H_2O_2-treated Jurkat cell extracts revealed that particularly after three hours of oxidative stress important changes were observed, which at least in part will be due to the induction of necrosis as indicated by the appearance of the specific topoisomerase I cleavage product, but to oxidative protein modifications as well, as is evident from the western blot analyses. The number of proteins that appear to become modified upon H_2O_2 exposure implies that oxidative modification is not a unique feature of autoantigenic proteins. Remarkably, the modifications of Ro60, Jo-1, Rrp41, and U1A did not occur simultaneously, and, although differences in the rate by which these proteins are oxidized clearly exist, all autoantigenic proteins analyzed appeared to be modified prior to the initiation of the major changes affecting the total protein pattern. It will be interesting to investigate whether autoantigenic proteins tend to be modified relatively fast (Fig. 2). Importantly, only concentrations of H_2O_2 that are able to induce necrosis result in oxidatively modified proteins, which implies that the modifications may also be caused by necrosis-associated activities. Another open question is whether the oxidatively modified autoantigenic proteins, in contrast to other proteins, are exposed to the immune system and lead to a modification-dependent immune response. For such studies, techniques that allow the specific detection of modified epitope targeting autoantibodies are needed.

Since H_2O_2-induced necrosis might be involved in the observed modifications, the possibility existed that intracellular ROS (e.g. from mitochondria) played a role. However, none of the alternative necrosis induction methods (e.g. the exposure to ethanol, $HgCl_2$ or heat) [24-26], which are associated with mitochondrial damage, nor generators of intracellular superoxide radicals [19,20] led to modifications with

a similar retarded electrophoretic mobility (data not shown and Fig. 5). Because the alternative necrosis induction methods, through mitochondrial damage, also mainly generate intracellular superoxide anion radicals, we conclude that superoxide radicals do not play a major role in the oxidative modification of the autoantigenic proteins. The hydroxyl radical, $OH^{\bullet}$, on the other hand, appears to be strongly involved in the oxidative modification of the proteins studied here, since both the *in vitro* oxidation of recombinant proteins and the treatment of Jurkat cells or recombinant proteins with NaOCl resulted in a mobility shift similar to that observed in H_2O_2 treated material (Fig. 4 & 6). The exact chemical nature of the modifications caused by the exposure to hydroxyl radicals requires further investigation, e.g. by the isolation of modified proteins and their analysis by mass spectrometry.

Taken together, our data show that the exposure to hydroxyl radicals leads to rapid oxidative modifications of autoantigenic proteins, which can be monitored via their altered electrophoretic mobility in SDS-PAGE gels. These modifications are probably not unique for autoantigenic proteins, although differences in the rate of modification may exist. The questions whether autoantigenic proteins are generally modified faster than non-autoantigenic proteins and whether the oxidative modifications lead to the formation of neo-epitopes, require further investigations.

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Chapter 4

Oxidative stress-induced modifications of histidyl-tRNA synthetase affect its tRNA aminoacylation activity but not its immunoreactivity

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Abstract

The aminoacyl-tRNA synthetases (aaRS) are ubiquitously expressed enzymes that catalyze the esterification of amino acids to their cognate tRNAs. Autoantibodies against several aaRS are found in autoimmune polymyositis and dermatomyositis patients. Because necrosis is often found in skeletal muscle biopsies of these patients, we hypothesized that cell death-induced protein modifications may help in breaking immunological tolerance. Since cell death is associated with oxidative stress, the effect of oxidative stress on the main myositis-specific autoantibody target Jo-1 (histidyl-tRNA synthetase; HisRS) was studied in detail. The exposure of Jurkat cells to hydrogen peroxide resulted in the detection of several oxidized methionines and one oxidized tryptophan residue in the HisRS protein, as demonstrated by mass spectrometry. Unexpectedly, the tRNA aminoacylation activity of HisRS appeared to be increased upon oxidative modification. The analysis of myositis patient sera did not lead to the detection of autoantibodies that are specifically reactive with the modified HisRS protein. The results of this study demonstrate that the Jo-1/HisRS autoantigen is modified under oxidative stress conditions. The consequences of these modifications for the function of HisRS and its autoantigenicity are discussed.

Introduction

Aminoacylation of tRNAs plays a crucial role in the translation of genetic information in polypeptide chains. The generation of aminoacylated-tRNAs depends on the ubiquitously expressed aminoacyl-tRNA synthetases (aaRS), which conjugate amino acids to their cognate tRNAs via an esterification reaction. In patients with idiopathic inflammatory myopathies (IIM / myositis), particularly polymyositis (PM) and dermatomyositis (DM), autoantibodies against several aaRS are found [1,2]. The IIM are a group of systemic autoimmune disorders that are associated with inflammation of skeletal muscles [3-5].

Autoantibodies directed to intracellular proteins, which can be divided into myositis-specific autoantibodies (MSA) and myositis-associated autoantibodies (MAA), are found in up to 80% of patients with PM or DM [6]. MAA are also found in other rheumatic disorders. MSA include autoantibodies directed against a component of the nucleosome remodeling and deacetylase complex, Mi-2, the signal recognition particle (SRP) and several aaRS [7,8]. The most prevalent MSA is directed against the histidyl-tRNA synthetase, HisRS (the so-called anti-Jo-1 antibody), and can be detected in approximately 20% of PM/DM patients. Autoantibodies directed to other tRNA synthetases collectively are found in about 20% of myositis patients [9,10]. Environmental, genetic, and hormonal factors, together with the loss of immunological tolerance, play an important role. Inflammatory processes have been suggested to deregulate the maintenance of tissue homeostasis and immunological tolerance. Slight aberrations in apoptotic clearance mechanisms, like in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have a great impact on the physiological processes of the immune system [11-13]. An increasing number of studies show that post-translational modifications of nuclear and cytoplasmic components can be involved in the breaking of immunological tolerance. Specific polypeptide cleavages and/or unusual amino acid modifications occur during apoptotic or necrotic cell death, and have been suggested to be involved in the generation of neo-epitopes that are presented to the immune system [13-15]. In view of the autoimmunity associated with myositis, the infiltration of inflammatory cells in the affected muscles, and the degeneration of muscle fibers, it is of great interest to investigate cell death-induced autoantigen modifications. Here, we studied apoptosis and necrosis-induced modifications of the major autoantigen in myositis, histidyl-tRNA synthetase (Jo-1). We focused on post-translational modifications possibly occurring in the pathophysiological microenvironment found in myositis patients [16]. It has been shown that massive production of oxidizing agents occurs upon myocyte damage, suggesting an important role of reactive oxygen species (ROS) during muscle trauma [17]. Both mechanical damage and T-lymphocytes are able to induce muscle trauma. Because human skeletal muscle cell lines were not readily available and because the induction of cell death in cultured mouse myoblasts and myotubes did not proceed in a very coordinated fashion, we decided to use Jurkat cells (a human T lymphoblastoid cell line), to study modifications of HisRS under oxidative conditions. An increased oxidation status of the HisRS protein in oxidatively stressed Jurkat cells was observed, which unexpectedly increased its aminoacylation activity. The potential relevance of these modifications for the generation of anti-Jo-1 antibodies is discussed.

Materials and methods

Cell lines

Jurkat (human T cell leukemia) cells were grown in RPMI 1640 Glutamax™-I medium supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin (Gibco) and 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH). HEp-2 cells were grown to 80% confluency in DMEM$^+$ (Gibco) containing 1 mM penicillin, 1 mM streptomycin and 10% heat-inactivated fetal calf serum. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Induction of cell death and cell extract preparation

Jurkat cells were maintained at a concentration of 10^6 cells/ml. Induction of apoptosis was accomplished by the addition of 40 µM anisomycin or various concentrations of camptothecin as indicated (Sigma-Aldrich), followed by 8 hours incubation at 37 °C. For the induction of necrosis, cells were exposed to 0.1% H_2O_2 and incubated for various time points at 37 °C. Cells were harvested by centrifugation at 1,000 *g* for 5 min, and washed with PBS to remove residual medium. Cell pellets were resuspended in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM DTT and 1% Nonidet-P40 (NP-40) and lysed by sonication on ice. Subsequently, cell suspensions were cleared from insoluble material by centrifugation at 16,000 *g* for 30 min at 4 °C. Cell lysates were stored at -20 °C.

Patient sera and antibodies

Patients were diagnosed with PM or DM at the

Department of Neurology of the University Medical Centre Nijmegen (The Netherlands) according to standard criteria [18,19]. The PM/DM patient sera were obtained with informed consent of all participants and stored at -70 °C until use. Myositis patient sera with reactivity against the alanyl- (PL12) and threonyl-tRNA syntethase (PL7) were a kind gift of Prof. Dr. N. McHugh (Royal National Hospital for Rheumatic Diseases, University of Bath, UK). Polyclonal anti-glutamyl-prolyl-tRNA synthetase and monoclonal anti-methionyl-tRNA synthetase antibodies were purchased from Abcam (Cambridge, UK). The single chain monoclonal antibody (scFv) directed to HisRS has been described previously [20]. The monoclonal anti-eIF2α antibody has been described before [21] and the anti-ß-actin antibody was purchased from Sigma-Aldrich.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Sample buffer, containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercapto-ethanol, 10% glycerol and 0.005% Bromophenol Blue, was added to an equal volume of cell lysate. After heating at 95 °C for 5 min material from $0.5*10^6$ Jurkat cells per lane was loaded on 10% SDS-polyacrylamide gels and after electrophoresis transferred to nitrocellulose membranes by semi-dry western blotting. Protein transfer was checked by Ponceau S (Sigma-Aldrich) staining of the membrane. All further immunoblotting steps were performed under agitation at room temperature. Membranes were blocked with 3% non-fat dried milk in PBS containing 0.05% Tween-20 (Fluka BioChemika; MPBST). Patient serum in a 2,500-fold dilution or the anti-HisRS scFv (10-fold diluted) in MPBST was added and bound antibodies were detected with IRDye^{800}-labeled goat anti-human or goat anti-mouse antibodies (LI-COR), 2,500-fold diluted in MPBST, followed by near-infrared detection and quantification with Odyssey 2.1 software and equipment.

Affinity purification

Anti-Jo-1 autoantibodies were isolated from patient sera by affinity chromatography using immobilized recombinant human HisRS protein expressed in *E. coli* (BL21) [22]. The isolated antibodies were covalently coupled to CNBr-activated Sepharose 4B (Amersham Bioscience) and used for the purification of the HisRS protein from healthy and necrotic Jurkat cells. Anti-Jo-1-Sepharose was equilibrated with IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Tween-20, 1 mM DTT and 0.1% NP-40). Cleared cell lysates, centrifuged at 16,000 *g* for 15 min at 4 °C, were added and incubated for 4 h in an end-over-end rotor at 4 °C. To minimize non-specific adsorption, a high salt elution step with IPP1000 (1 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Tween-20, 1 mM DTT and 0.1% NP-40) was included prior to the elution of the bound HisRS protein by a pH shock (0.1 M glycine-HCl, pH 2.5). Eluted material was neutralized immediately with Tris-base. The purified HisRS preparations were stored at -20 °C until use.

Depletion of anti-Jo-1 reactivity to recombinant human HisRS

Anti-Jo-1 reactivity in patient sera against the bacterially expressed recombinant human HisRS protein was removed by pre-incubating the anti-Jo-1 sera with 0.1, 1, 10, or 100 ng purified recombinant HisRS in 100 µl of 400- to 50,000-fold diluted serum in PBS. After incubation for 30 min at room temperature, sera were centrifuged at 16,000 *g* for 30 min at 4 °C. The anti-Jo-1 depleted and the untreated control anti-Jo-1 sera were used for immunoblotting as described above.

Analysis of HisRS oxidation by LC-MS/MS

Recombinant human HisRS was oxidized *in vitro* by incubation in a solution containing 1.7 mM ascorbic acid (Fluka BioChemika), 0.1 mM $Fe(II)SO_4$ (Merck) and 0.18% H_2O_2 for 10 min at room temperature. The reaction was stopped by the addition of 1 mM EDTA (final concentration). Recombinant HisRS and *in vitro* oxidized HisRS, as well as HisRS isolated from healthy Jurkat cells and from oxidatively stressed Jurkat cells (see above) were separated by 10% SDS-PAGE and stained using colloidal Coomassie Brilliant Blue (CBB; 0.08% CBB G250, 1.6% ortho-phosphoric acid, 8% ammonium sulfate and 20% methanol). The bands corresponding to HisRS were excised from the gel and washed with water. Prior to in-gel digestion, proteins were reduced with 1,4-dithiothreitol (6.5 mM) and alkylated with iodoacetamide (54 mM). After thorough washing, pieces were rehydrated in trypsin solution (10 ng/µl) on ice. After addition of 30 µl of NH_4HCO_3 (50 mM, pH 8.5), samples were digested for 16 h at 37 °C. The supernatant of the digest was collected and the gel pieces were washed for 15 min in 5% formic acid at room temperature, after which the supernatant was combined with the earlier fraction and stored at –20 ºC. All LC-MS/MS analyses were performed on a LTQ-FTICR mass spectrometer (Thermo, San Jose, CA) connected to an Agilent 1100 series nano-LC system. Peptides were separated on C18 with a multi-step gradient of 0.6% acetic acid (HAc) and 0.6% HAc/80% acetonitrile. The mass spectrometer

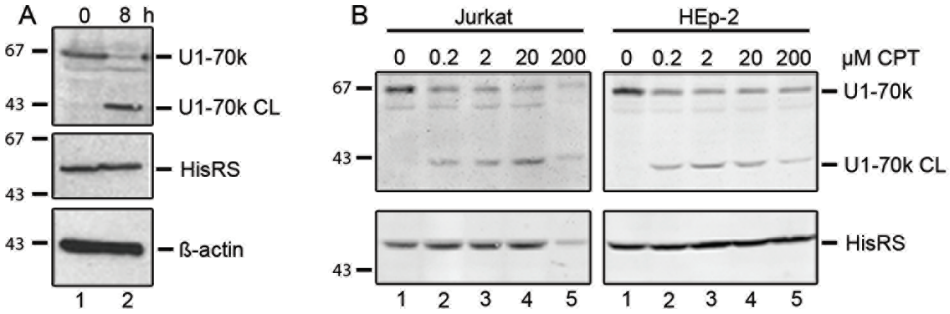

Fig. 1 Analysis of the HisRS autoantigen in apoptotic cells.
Jurkat or HEp-2 cells were treated with anisomycin or camptothecin, and cell lysates were analyzed by immunoblotting. Cleavage of the U1-70k protein leading to a 40-kDa product (U1-70k CL), was used to monitor apoptosis. (**A**) The HisRS protein was detected by an anti-Jo-1 positive patient serum in lysates from untreated and anisomycin-treated Jurkat cells (for 8 h). The detection of β-actin was included as a loading control. (**B**) Anti-Jo-1 antibodies were used to detect HisRS in lysates from Jurkat and HEp-2 cells treated for 8 hours with various concentrations of camptothecin (CPT). The positions of molecular weight markers are indicated on the left.

was operated in the data-dependent mode to automatically switch between MS and MS/MS, without dynamic exclusion to allow accurate spectral counting. Raw MS data were converted to peak lists using Bioworks Browser software, version 3.1.1., and search against the IPI human (v3.36) database with a precursor mass tolerance of 10 ppm and a product mass tolerance of 0.9 Da. In several separate searches, various oxidative protein modifications were included as variable modifications, oxidation of M, Y, F, W, H, L, P, R, D and N; double oxidation of M and W, triple oxidation of W (see supplementary information), chlorination of Y and nitration of Y. To obtain quantitative information, spectral counting was performed after a final search including only oxidation of M, D, F, Y and W, double oxidation of M and W and triple oxidation of W as variable modifications. The minimum Mascot score accepted for identification of an oxidized peptide was 30, the minimum Mascot score for identified spectra included in the spectral count was 20.

In vitro aminoacylation assay

Healthy and H_2O_2-treated (2 h) Jurkat cells were harvested by centrifugation, 1,000 *g* for 5 min at room temperature, resuspended in MilliQ water supplemented with 1 mM DTT and lysed by 5 30-second pulses in an ice-cold sonication waterbath (Bioruptor). The protein suspensions were cleared by centrifugation at 21,000 *g* for 30 min at 4 °C. Twenty million Jurkat cell equivalents were used in a 50 µl aminoacylation mixture, containing 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 20 mM Tris-HCl, pH 7.4, 15 µM [^{14}C]histidine (Amersham), and 2.5 U/µl RNasin (Promega), and incubated for 1.5 hours at 37 °C. Prior to RNA separation on a 6.5% polyacrylamide, 0.1 mM NaOAc, pH 5.0, 7.5 M urea gel system, the RNA was isolated from the aminoacylation mixture using TRizol [23]. After electrophoresis, RNA was transferred to Hybond-N membranes (Amersham) and visualized by phosphorimaging. Total $tRNA^{His}$ and 5.8S rRNA were visualized by northern blot hybridization using [^{32}P]-radiolabeled antisense probes as described [24]. The hybridization with the antisense $tRNA^{His}$ or 5.8S rRNA probes was performed overnight at 65 °C, in hybridization buffer (6x SSC, 1x Denhardt's, 0.2% SDS, and 0.1 mg/ml herring sperm DNA). Subsequently, membranes were washed twice with buffer containing 2x SSC and 0.1% SDS. The hybridization of probes was visualized by phosphorimaging and the signals were quantified by Quantity One software v4.6.9 (BioRad).

Results

HisRS modifications in apoptotic/necrotic human cell lines

Jurkat cells were cultured in the presence of anisomycin to induce apoptosis and in the presence of H_2O_2 to trigger necrosis (see Materials and methods). Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with autoimmune patient sera containing autoantibodies against U1-70k and topoisomerase I (Topo I), as biochemical markers of apoptosis and necrosis, respectively [25-27]. The appearance of the characteristic cleavage products of these proteins (Fig. 1A and 2A) confirm the induction of apoptosis and necrosis, respectively. No change in the electrophoretic mobility of the HisRS protein, which may be indicative of protein modification, was observed in material from apoptotic Jurkat cells (Fig. 1A). Also after induction of apoptosis in Jurkat or HEp-2 cells by camptothecin no changes in HisRS mobility were detected (Fig. 1B). In the

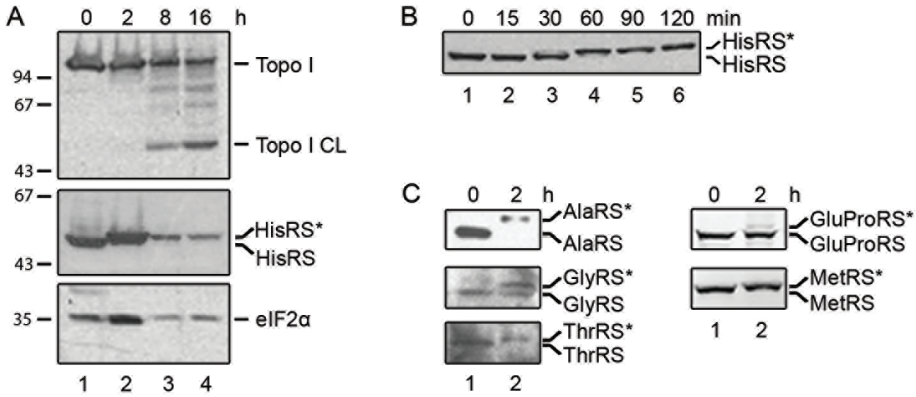

Fig. 2 Hydrogen peroxide induces HisRS modification.
(**A**) Jurkat cells were cultured in the presence of 0.1% H_2O_2 for the indicated periods of time. Cell lysates were analyzed by immunoblotting using antibodies to Topoisomerase I (Topo I) to monitor the induction of necrosis, which is indicated by the appearance of a 45-kDa cleavage product (Topo I CL). HisRS was detected by anti-Jo-1 antibodies and eIF2α served as a loading control. The position of HisRS in the lysates from H_2O_2-treated cells is indicated by HisRS*. (**B**) The cells were cultured for 0 – 120 min with 0.1% H_2O_2, before they were lysed and analyzed by immunoblotting with anti-Jo-1 antibodies. (**C**) Immunoblotting analysis of normal (0 h) and H_2O_2–treated (2 h) Jurkat cell lysates using antibodies to other autoantigenic aminoacyl-tRNA synthetases (AlaRS, GlyRS, ThrRS) as well as to non-autoantigenic (GluProRS and MetRS) aminoacyl-tRNA synthetases.

necrotic cell extracts we observed a reduction of the electrophoretic mobility of the HisRS protein (HisRS*) that already could be detected 30 minutes after adding H_2O_2, which is several hours before the first detection of Topo I cleavage (Fig. 2A, B). Eight hours after the induction of necrosis the intensity of the HisRS signals decreased, which is most likely due to cell death and resulting in the leakage of intracellular content, or to accelerated degradation of the modified HisRS protein. The loss of cytosolic porteins as a result of cell death is supported by a similar reduction in the intensity of the signals of the translation initiation factor eIF2α, which served as a loading control (Fig. 2A). The analysis of lysates from other cell lines such as 3T3, a mouse fibroblast, COS-1, an African green monkey kidney fibroblast, HEp-2, a human epithelial cell line, TE671, a human rhabdomyosarcoma cell line, C2, a mouse myoblast, and HSMM, a human skeletal muscle myoblast showed a similar change in electrophoretic mobility of HisRS upon H_2O_2 treatment (data not shown). An altered migration pattern was also detected for other autoantigenic aaRS (glycyl- (EJ), threonyl- (PL7) and alanyl-tRNA synthetase (PL12)) and for the non-autoantigenic glutamyl-prolyl-tRNA synthetase (GluProRS) and methionyl-tRNA synthetase (MetRS, Fig. 2C), although for the latter synthetases the efficiency of modification appeared to be much lower than that of the autoantigenic aaRS. Other necrosis-inducing methods such as exposure to 0.2 mM $HgCl_2$, freezing-thawing, and the exposure to 10% ethanol, as well as anisomycin-induced secondary necrosis (all with Jurkat cells) did not result in changes in HisRS migration in SDS-PAGE gels (data not shown).

Affinity purification and characterization of modified HisRS

Hydrogen peroxide is a powerful oxidizing agent that can be converted to hydroxyl radicals with an even higher reactivity. This implies that the observed changes in HisRS migration might be caused by oxidative modification of specific amino acid side chains. To identify such amino acids the modified protein was isolated, proteolytically cleaved and analyzed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). Affinity-purified patient anti-Jo-1 autoantibodies were immobilized by covalent coupling to Sepharose and used to isolate the HisRS protein from the lysates of mock-treated as well as oxidatively stressed Jurkat cells. In parallel, bacterially expressed recombinant human HisRS protein (recHisRS) was subjected to oxidative conditions by generating hydroxyl radicals in solution by the Fenton reaction (see Materials and methods). HisRS from healthy Jurkat and necrotic Jurkat cells, and the untreated and oxidized recHisRS were separated by SDS-PAGE and stained using colloidal Coomassie prior to in-gel digestion with trypsin. The tryptic digests were then subjected to high resolution LC-MS/MS on an LTQ-FTICR mass spectrometer and the resulting mass spectra were searched against all human proteins, to identify any peptides containing oxidized amino acids. The total sequence coverage of HisRS was 72-76% for the protein isolated from the Jurkat cells and 81-84% for the recHisRS protein.

A

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  1 MAERAALEEL VKLQGERVRG LKQQKASAEL IEEEVAKLLK LKAQLGPDES KQKFVLKTPK
 61 GTRDYSPRQM AVREKVFDVI IRCFKRHGAE VIDTPVFELK ETLMGKYGED SKLIYDLKDQ
121 GGELLSLRYD LTVPFARYLA MNKLTNIKRY HIAKVYRRDN PAMTRGRYRE FYQCDFDIAG
181 NFDPMIPDAE CLKIMCEILS SLQIGDFLVK VNDRRILDGM FAICGVSDSK FRTICSSVDK
241 LDKVSWEEVK NEMVGEKGLA PEVADRIGDY VQQHGGVSLV EQLLQDPKLS QNKQALEGLG
301 DLKLLFEYLT LFGIDDKISF DLSLARGLDY YTGVIYEAVL LQTPAQAGEE PLGVGSVAAG
361 GRYDGLVGMF DPKGRKVPCV GLSIGVERIF SIVEQRLEAL EEKIRTTETQ VLVASAQKKL
421 LEERLKLVSE LWDAGIKAEL LYKKNPKLLN QLQYCEEAGI PLVAIIGEQE LKDGVIKLRS
481 VTSREEVDVR REDLVEEIKR RTGQPLCIC
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B

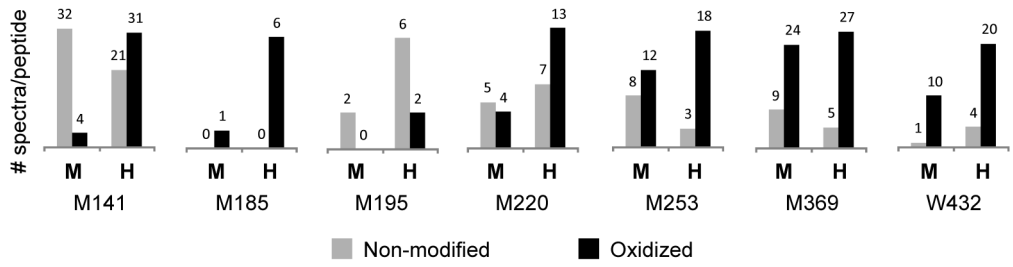


Fig. 3 Identification of oxidized residues in HisRS from H_2O_2–treated cells.
(**A**) Amino acid sequence of the HisRS protein, with the residues found to be oxidized in HisRS* highlighted by black boxes. Parts of the sequence covered by the MS/MS analysis are indicated in bold. (**B**) Quantitative comparison of the degree of oxidation of the seven oxidized HisRS residues from either untreated (M) or H_2O_2–treated (H) cells. The graph shows the number of identified mass spectra corresponding to peptides containing the non-modified form of the amino acid (in grey) and of spectra corresponding to an oxidized form of the indicated amino acid (in black). Numbers above the bars indicate the actual number of spectra identified.

A large number of distinct oxidative amino acid modifications were considered in several rounds of database searching, resulting in the confident identification (Mascot score >30) of oxidation sites across all samples. Oxidized methionine (M), tryptophan (W), and phenylalanine residues were identified. Seven oxidized residues (M141, M185, M195, M220, M253, M369 and W432; Fig. 3A) were observed in the HisRS protein from oxidatively stressed Jurkat cells and each of these was also found in the oxidized recHisRS protein (recHisRS*; data not shown). Representative spectra identifying the different peptides with oxidized residues are supplied in Fig. S1. The (untreated) recombinant protein contained additional oxidized residues, which were not detected in HisRS from unstressed or H_2O_2-stressed Jurkat cells and which suggests that additional amino acid oxidations occurred in vitro, e.g. during purification of recHisRS. As oxidative modifications can also occur on proteins not exposed to excessive oxidative stress and can be generated during sample preparation [28,29], we quantitatively assessed the occurrence of the oxidized residues in HisRS* as compared to HisRS isolated from mock-treated cells. By using spectral counting, several of the oxidation sites (especially M141, M220 and M253) were found to be significantly more present in HisRS*, whereas other residues were found to be oxidized to the same extent in both samples (Fig. 3B). The oxidized methionine residues were only reliably identified in their single oxidized form (methionine sulphoxide) and not as the double oxidized methionine sulphone, whereas different oxidized forms of W432 in the HisRS* protein were found (Fig. S2).

Aminoacylation activity upon oxidative modification of HisRS

Protein modifications most likely affect not only structural but also functional properties of proteins. To investigate the consequences of the oxidative modifications on the activity of HisRS, we determined the aminoacylation of histidyl-tRNA using extracts from healthy and H_2O_2-treated Jurkat cells. Radiolabeled ([^{14}C]) histidine was added to these extracts and after incubation the conjugation of [^{14}C]histidine to its cognate tRNA was determined by the isolation of RNAs from the reaction mixture and analysis of the reaction products by denaturing gel electrophoresis followed by phosphorimaging. The results showed that under these conditions the endogenous histidyl-tRNA was indeed aminoacylated and, more importantly, that the oxidative modification of HisRS did not abolish its activity. Interestingly, the histidyl-tRNA synthetase activity in H_2O_2-treated Jurkat cells appeared to be 1.7-fold higher as compared to mock treated cells (Fig. 4), which varied from 1.5- to

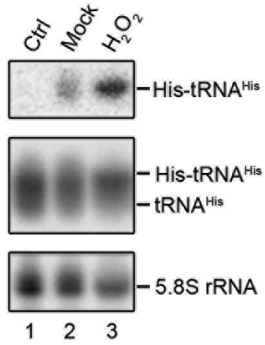


Fig. 4 Aminoacylation of histidyl-tRNA in extracts from H_2O_2–treated Jurkat cells.
Extracts from mock-treated (Mock) or H_2O_2-treated (H_2O_2) Jurkat cells were used in a tRNA aminoacylation assay (see Materials and methods). Aminoacylation was monitored by the conjugation of [^{14}C]histidine to tRNA^{His}. As a control the reaction was performed in the presence of a 1,000-fold excess of unlabeled histidine (Ctrl). Reaction products were analyzed by denaturing gel electrophoresis and phosphorimaging. The tRNA^{His} and 5.8S rRNA were detected by northern blot hybridization using [^{32}P]-labeled probes. Upper panel: [^{14}C] signals; middle and lower panels: northern blot signals for tRNA^{His} and 5.8S rRNA, respectively.

1.9-fold higher levels between three independent experiments. As a control, an excess of non-labeled histidine was added to the reaction and this indeed interfered with the conjugation of radiolabeled histidine. The presence of (non-)conjugated histidyl-tRNA in each of the reaction products was monitored by northern blot hybridization using an *in vitro* transcribed antisense probe. The results showed that the total amount of histidyl-tRNA did not significantly differ between these samples.

Recognition of HisRS by autoantibodies in myositis patient sera*

As a first approach to investigate the reactivity of patient anti-Jo-1 autoantibodies with the modified protein, synthetic peptides were generated based upon the histidyl-tRNA synthetase sequence and containing either methionine or methionine sulphoxide at the positions identified by mass spectrometry. The recognition of these 13-residue peptides corresponding to oxidation sites M141, M220, and M253 by patient antibodies was determined by ELISA. None of these peptides appeared to be reactive with 26 myositis sera, irrespective of the methionine oxidation state (results not shown).

Because the lack of reactivity with the synthetic peptides might be due to conformational limitations, we next analyzed the recognition of the complete (modified) HisRS protein on western blots by patient sera. Both oxidized as well as control HisRS protein was detected by patient antibodies. To be able to detect antibodies specifically targeting epitopes

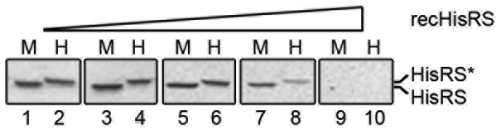


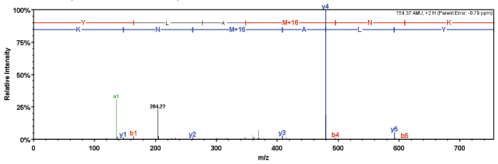
Fig. 5 Autoantibody reactivity in patient sera to HisRS modified in H_2O_2–treated Jurkat cells.
A diluted anti-Jo-1 positive patient serum was pre-incubated with an increasing amount of recombinant protein (recHisRS; lanes 1-2: no recHisRS added; lanes 3-4; 0.1 ng recHiRS added; lanes 5-6; 1 ng recHisRS added; lanes 7-8; 10 ng recHisRS added; lanes 9-10; 100 ng recHisRS added). Western blots containing lysates from mock- (M) and H_2O_2–treated (H) Jurkat cells were subsequently incubated with these serum samples. Bound antibodies were visualized by secondary antibody conjugates.

generated by the oxidative modifications, the sera were depleted of autoantibodies directed to the non-modified HisRS protein prior to their incubation with the western blots by adding increasing amounts of purified recHisRS protein. Western blots containing extracts from healthy and H_2O_2-treated Jurkat cells were incubated in parallel with normal and depleted myositis sera containing anti-Jo-1 antibody. The results in Fig. 5 illustrate that when a relatively small amount of serum is used together with an increasing amount of recHisRS a gradual depletion of autoantibodies was observed. Thirty-five anti-Jo-1 positive myositis sera, 9 sera from very early myositis patients, as well as 16 anti-Jo-1 negative myositis sera were screened for the presence of specific autoantibodies directed to HisRS* by this approach, but in none of these cases a signal that was resistant to depletion with recHisRS remained. Therefore, this approach did not lead to the detection of modification-specific anti-Jo-1 autoantibodies.

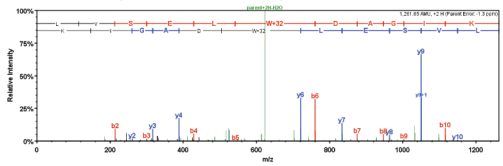
Discussion

Myositis is a relatively rare disease that severely impairs the patients' muscular system. Skeletal muscle dysfunction associated with mononuclear cell infiltration is commonly found in myositis patients. These local infiltrates are capable of generating high amounts of reactive oxygen/nitrogen species (e.g. during a respiratory burst), thereby inducing cell death mechanisms (e.g. apoptosis or necrosis) in healthy muscle tissue. Inflammation-induced cell death may result in post-translational modifications that could break immunological tolerance. However, continued stimulation of the immune system is needed for the development of autoantibodies and autoimmunity. Recently, Casciola-Rosen and collaborators found that regenerating myocytes in myositis patients express higher levels of autoantigenic proteins

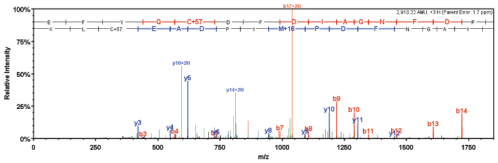
YLAMNK (M141 oxidized), Mascot score 34



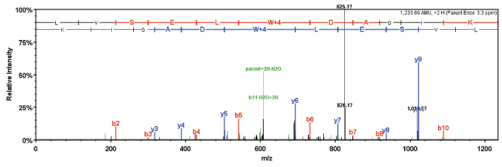
LVSELWDAGIK (W432 double oxidized), Mascot score 58



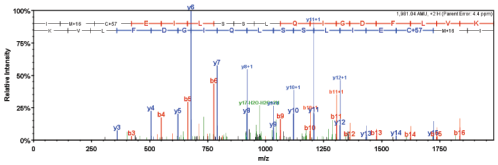
EFYQCDFDIAGNFDPMIPDAEcLK (M185 oxidized),
Mascot score 60



LVSELWDAGIK (W432 triple oxidized), Mascot score 52

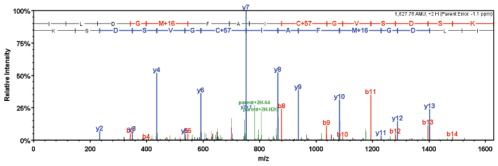


IMCEILSSLQIGDFLVK (M195 oxidized), Mascot score 56

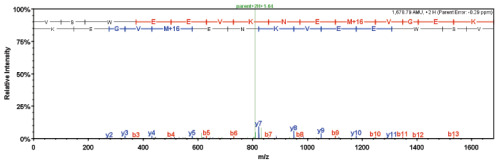


Supplementary Fig. 1 Identification of all oxidation events in HisRS from H_2O_2-treated Jurkat cells.
MS/MS spectra obtained with HisRS isolated from H2O2-treated Jurkat cells, which allowed the unequivocal identification of oxidation events on methionines M141, M185, M195, M220, M253, M368, and on tryptophan W432. Indicated are the peaks used for identification and the Mascot score with which this peptide was identified.

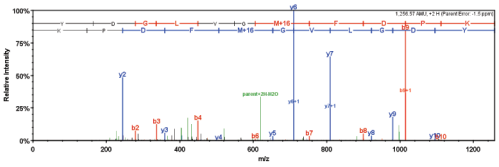
ILDGMFAICGVSDSK (M220 oxidized), Mascot score 107



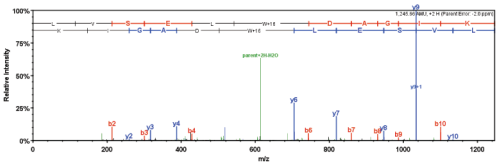
VSWEEVKNEMVGEK (M253 oxidized), Mascot score 53



YDGLVGMFDPK (M369 oxidized), Mascot score 53

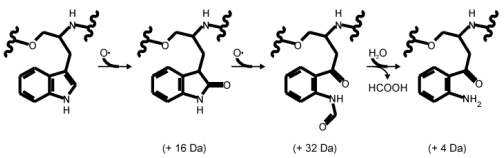


LVSELWDAGIK (W432 oxidized), Mascot score 60



(including Jo-1/HisRS) compared to tissue from healthy controls. They suggested that this process might perpetuate the chronic inflammatory cycle [30]. Up to now only a few HisRS modifications have been described in literature. Ramirez-Sandoval and colleagues described the appearance of a HisRS cleavage product of 53 kDa upon campothecin-mediated induction of apoptosis in HEp-2 cells [31]. We did not observe a similar cleavage product upon the induction of apoptosis, neither in Jurkat nor in HEp-2 cells treated with camptothecin or with anisomycin (Fig. 1A, B). In addition, HisRS has been reported to be cleaved by Granzyme B, which is one of the serine proteases that are present in the granules of cytotoxic T-cells and natural killer cells [32]. These HisRS fragments are able to recruit mononuclear cells *in vitro*, but whether this represents the situation at the damaged site and results in the initiation of innate and adaptive immune responses associated with the development of myositis still has to be investigated [33].

Our results demonstrate that HisRS and probably also other autoantigenic and non-autoantigenic aaRS are oxidatively modified in cell lines treated with H_2O_2. Hydrogen peroxide has been used by several researchers to study necrosis and this showed a certain cell death specificity [27]. Apoptotic pathways were activated by low levels of H_2O_2, whereas necrotic characteristics were observed in cells that were exposed to relatively high levels of H_2O_2. In addition to being an established necrosis-inducing compound H_2O_2 leads to the formation of radicals that are also produced by infiltrating immune cells and during tissue damage in the microenvironment found in myositis patients' muscles [16]. The hydroxyl



Supplementary Fig. 2 Oxidative modification of tryptophan.
Schematic representattion of the oxidative modification of tryptophan (left-most structure) to its oxidized form (which is 16 Da heavier), to formylkynurenine (32 Da heavier) and to kynurenine (4 Da heavier).

radical is among the most potent oxidizing agents that are generated from H_2O_2, and can also be released by inflammatory cells infiltrating (muscle) tissues [34,35]. Resting skeletal muscle cells have been shown to release low amounts of superoxide anion and H_2O_2 to the extracellular environment. These amounts may increase considerably with enhanced muscle activity [36,37]. Studies of Kerkweg and colleagues showed increased superoxide release immediately after myocyte damage [17], suggesting an important role of ROS in signaling mechanisms during tissue trauma. In order to discriminate between oxidative stress-mediated or necrosis-induced modifications, Jurkat cells were subjected to other necrotizing stimuli, like ethanol, freezing-thawing, $HgCl_2$, and anisomycin-induced secondary necrosis. None of the alternative necrosis-inducing methods resulted in a similar retarded HisRS migration as observed with H_2O_2-treated cells, suggesting that the observed HisRS modifications are the result of oxidative stress. The modification of HisRS appeared to be virtually complete two hours after the addition of H_2O_2, whereas at that time-point hardly any necrosis-specific cleavage of Topoisomerase I could be detected. These results suggest that the detected HisRS modifications are not a general necrotic phenomenon but might be a specific result of oxidative stress-induced necrosis.

High resolution LC-MS/MS analysis of the purified modified protein allowed the identification of several oxidation sites (M141, M185, M195, M220, M253, M369, and W432). The highest complexity of oxidation products was observed for W432 in both HisRS* and recHisRS* (Fig. S1). The oxidation of tryptophan is known to consist of three sequential steps resulting in the formation of oxidized tryptophan, N'-formylkynurenine, and kynurenine, respectively [38] (Fig. S2). The presence of (formyl)kynurenine has been described in mitochondrial proteins, but has so far never been linked to an autoantigen or to autoimmunity [39]. The oxidative HisRS modifications might generate epitopes recognized by the immune system. Several epitope mapping reports determined autoantibody activities directed against specific regions of the HisRS protein. Martin and colleagues described reactivities of anti-Jo-1 positive myositis sera against two amino acid stretches, namely 2-44 and 286-509, in a HisRS fragment study covering the full-length HisRS amino acid sequence [40]. Two oxidation sites (M369, W432) found in our study might be related to epitopes found in the latter protein fragment and might hint to the origin of autoantibody reactivity that has been described before. Oxidatively modified proteins have already been implicated in the development of autoimmune diseases like systemic sclerosis (Topoisomerase I) and type 1 diabetes (GAD65) [41,42]. Our analyses with established myositis patient sera did not reveal autoantibodies specifically targeting HisRS epitopes generated by the oxidative modifications. Since such antibodies are hypothesized to be among the first that are produced upon the induction of autoimmunity, we also analyzed whether the modified HisRS protein is recognized by very early and/or 'pre-disease' sera. However, in 9 very early anti-Jo-1 positive myositis sera such antibodies could not be detected. Our results thus indicate that the oxidative modifications of HisRS are not directly linked to the B-cell response in myositis. Alternatively, the procedures applied to detect modification-dependent antibody reactivities might not be optimal. A disadvantage of synthetic peptides is that they are largely unstructured and will not allow the detection of antibodies targeting discontinuous epitopes. The immunoblot procedure in combination with the depletion of antibodies binding to the bacterially expressed recombinant protein may not be suitable for the detection of reactivity to discontinuous and/or conformational epitopes, because the refolding of the immobilized protein on the blot might be far from optimal. In addition, the recombinant protein used for the depletion may not be completely devoid of oxidative modifications, which could lead to the depletion of modification-specific anti-Jo-1 antibodies. To confirm that indeed oxidative modification are not recognized by autoantibodies directed to HisRS further studies using other procedures and materials, e.g. sandwich ELISA, and very early or 'pre-disease' patient samples will be required. Alternatively, immunoprecipitation from radiolabeled cell extracts or in combination with immunoblotting may provide opportunities to determine whether specific anti-HisRS* autoantibodies do exist or not. The latter immunoprecipitation approach with patient sera is, however, not possible, because the immunoglobulin heavy chains comigrate with HisRS in SDS-PAGE. In addition, also in immunoprecipitation the reactivity of the polyclonal patient sera with HisRS epitopes that

are not modified under oxidative conditions would have to be blocked to allow the specific detection of reactivity with the modified epitopes. The addition of the recombinant HisRS protein to achieve this is not possible, because this would lead to co-precipitation of the recombinant protein.

Oxidation of amino acids can have several consequences for the function of the protein [43,44]. Surprisingly, oxidative modification of HisRS did not detectably impair its aminoacylation activity. Instead, the efficiency by which histidyl-tRNA was aminoacylated appeared to be slightly increased. Pairwise alignment of the human HisRS amino acid sequence with that of histidyl-tRNA synthetases from other organisms, for which structural information is available in the PDB database, revealed that M141, M220 and M253 do not seem to be located in regions that are critically important for the activity of the enzyme. The enhanced activity might be explained by stabilization of the enzymatically active form or by an increased affinity for substrate molecules.

Acknowledgements

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Chapter 5

Oxidative stress-induced intermolecular disulfide bonds in the histidyl-tRNA synthetase homodimer

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Abstract

The aminoacyl-tRNA synthetases are conserved throughout all forms of life and catalyze the aminoacylation of tRNAs, a critical step in the translation of genetic information. The aminoacyl-tRNA synthetases can be divided in two classes, depending on sequence phylogeny, conserved sequence motifs, and aminoacylation activities. Recently, oxidative stress has been implicated in the regulation of the function of several intracellular proteins, and to affect aminoacylation activities. Here, we show that a covalent bond is formed between the two monomers of the dimeric histidyl-tRNA synthetase (HisRS) in oxidatively stressed cells. The covalent dimerization appeared to be sensitive to reducing agents, suggesting the involvement of disulfide bonds. The isolation of the crosslinked dimer followed by its analysis by mass spectrometry allowed the identification of four cysteines involved in disulfide bond formation. In combination with the results obtained with amino acid substitution mutants, two intermolecular disulfide bonds were mapped in the C-termini of both monomers. In spite of the oxidative modifications of HisRS, the histidyl-tRNA aminoacylation activity in extracts from oxidatively stressed cells was not abolished.

Introduction

The aminoacyl-tRNA synthetases (aaRSs) are found in all living organisms and catalyze the aminoacylation of tRNAs. The coupling of a particular amino acid to its cognate tRNA is an important step in translation. Eukaryotic cells contain an aaRS for each individual amino acid, except for the bifunctional glutamyl-prolyl-tRNA synthetase that takes care of coupling two amino acids to the corresponding tRNAs [1]. In addition, both cytosolic and mitochondrial forms of each aaRS are present in mammals [2]. The aminoacylation of tRNAs proceeds in a two step reaction: (1) the amino acid is adenylated via a reaction with ATP, which results in aminoacyladenylate and pyrophosphate, and (2) the adenylate is replaced by the terminal adenosine residue of the tRNA acceptor stem. These so-called charged tRNAs deliver the attached amino acid in a codon-dependent manner to the ribosome associated with the mRNA to be translated. The aaRSs are divided into two classes and three sub-classes based on their sequences, conserved sequence motifs, and aminoacylation activities. The catalytic domain of Class I aaRSs contains a Rossmann fold and two highly conserved sequence motifs whereas Class II aaRSs are characterized by an anti-parallel ß-sheet surrounded by α-helices and three conserved sequence motifs [3]. Additionally, Class I enzymes mainly form monomeric and dimeric complexes and aminoacylate the 2'-OH group of the terminal ribose of tRNA, after which the aminoacyl group is transferred to the 3'-OH by a transesterification reaction. Class II aaRS usually form dimeric and tetrameric structures and aminoacylate the 3'-OH group of the terminal ribose. All individual aaRSs, except lysyl-tRNA synthetase (LysRS), belong to either Class I or Class II throughout all forms of life. This high level of conservation of structural and functional characteristics emphasizes the importance of these enzymes in translation. However, during evolution many individual aaRSs developed distinct structural features, additional domains, and new biological functions in addition to their canonical activities [4]. The additional domains are suggested to facilitate the formation of a so-called multisynthetase complex, which can be found in higher eukaryotes and is thought to promote protein synthesis, proofreading activity, and serve as a reservoir of regulatory molecules that are involved in functions other than aminoacylation [5-7]. Recent investigations report a potential redox-dependent regulation of several aaRSs. The studies show how oxidative stress-induced modifications of critical cysteines in prokaryotic glutamyl-tRNA synthetase (GluRS) and threonyl-tRNA synthetase (ThrRS), are able to impair the aminoacylation activity [8,9]. On the other hand, Wakasugi and co-workers reported the importance of an N-terminal extension in mammalian tryptophan-tRNA synthetase (TrpRS), which is crucial for the interaction with oxidized GAPDH and the upregulation of its aminoacylation activity [10]. The N-terminal extension shares amino acid sequence homology with several otherwise non-homologous aaRSs. This so-called WHEP (TrpRS, HisRS, Glu/ProRS, and MetRS) domain mediates various (non-)canonical functions, including the assembly of a multisynthetase complex, tRNA binding and enzymatic activities [11-14]. We wondered whether human WHEP domain containing aaRSs are also regulated in a redox-dependent way. Histidyl-tRNA synthetase (HisRS) is a Class IIa aaRS containing three conserved domains as well as an N-terminal WHEP domain [15]. The conserved domains are involved in its catalytic activity, tRNA anticodon binding, tRNA acceptor stem interactions, and histidine adenylation [16-18]. Here, we describe the oxidative stress-induced formation of disulfide bonds in HisRS homodimers. Mass spectrometry, computational modeling and mutagenesis studies provided insight in the structural aspects of the dimerization and revealed the residues involved.

Materials and methods

Cell culture

Human T cell leukemia cells (Jurkat) were grown in RPMI 1640 Glutamax™-I medium supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin (Gibco) and 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Induction of oxidative stress and cell lysate preparation

Jurkat cells were maintained at a concentration of 10^6 cells/ml. Oxidative stress was induced by exposing cells for various periods to 0.1% H_2O_2 at 37 °C. Cell suspensions were harvested by centrifugation at 1,000g for 5 min, and washed with PBS. Cell pellets were resuspended in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 1% Nonidet-P40 (NP-40), with or without 1 mM DTT and lysed by sonication on ice. Insoluble material was removed by centrifugation at 21,000g for 30 min at 4 °C. Cleared cell lysates were used immediately.

Protein digestion for LC-MS/MS

Recombinant HisRS was separated by non-reducing 10% SDS-PAGE and stained using colloidal Coomassie Brilliant Blue staining (0.08% CBB G250, 1.6% ortho-phosphoric acid, 8% ammonium sulfate and 20% methanol). The band corresponding to dimerized HisRS was excised from the gel and washed with MilliQ water. To be able to determine cysteine residues involved in disulfide bridges, the protein was either reduced with 1,4-dithiothreitol (6.5 mM) and alkylated with iodoacetamide (54 mM) or directly alkylated with iodoacetamide. Following alkylation, the protein was in-gel digested with either trypsin or chymotrypsin (3 ng/µl) for 16 h at 37 °C. The supernatant of the digest was collected and the gel pieces were washed with 5% formic acid for 15 min at room temperature, after which the supernatants were combined and stored at –20º C.

Nano LC-MS/MS

All LC-MS/MS analyses were performed on a nanoLC LTQ-Orbitrap Velos (Thermo, San Jose, CA) mass spectrometer at a resolution of 60,000 at 400 m/z. For nanoLC, an Agilent 1200 series LC system was equipped with a 25 mm Aqua C18 (Phenomenex, Torrance, CA) trapping column (packed in-house, i.d., 100 µm; resin, 5 µm) and 400 mm ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) analytical column (packed in-house, i.d., 50 µm; resin, 3 µm). Solvents used where 0.6% HAc (buffer A) and 0.6% HAc/80% acetonitrile (ACN) (buffer B). Trapping was performed at 5 µL/min for 10 min, and elution was achieved with a gradient of 13–32% B in 30 minutes, 32-40% B in 5 minutes, 40-100% B in 2 minutes and 100%B for 2 minutes. The flow rate was passively split from 0.6 mL/min to 100 nL/min. Nanospray was achieved using in-house made distally coated fused silica emitters (o.d., 375 µm; i.d., 20 µm) biased to 1.8 kV. The mass spectrometer was operated in the data dependent mode to automatically switch between MS and MS/MS. Survey full scan MS spectra were acquired from m/z 350 to m/z 1500 and the two most intense ions were fragmented using CID, ETD (in the LTQ) and HCD (in the Orbitrap). The target ion setting was 5e5 for the Orbitrap, with a maximum fill time of 250 ms. Fragment ion spectra were acquired with a target ion setting of 5e3 and a maximum fill time of 100 ms in the LTQ and a target ion setting of 3e5 and a maximum fill time of 500 ms in the Orbitrap. The normalized collision energy was set to 35% for both CID and HCD. Supplemental activation was enabled for ETD. Dynamic exclusion for selected precursor ions was set at 30 seconds.

LC-MS/MS data processing and analysis

Raw MS data were converted to peak lists using Proteome Discoverer (v1.2). For protein and peptide identification, MS/MS data were searched against all human proteins in the Swissprot database, using Mascot v2.2 (Matrix Science) with the appropriate enzyme, allowing up to 2 miss cleavages. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methinione and tryptophan, double oxidation of methinione and tryptophan and triple oxidation of tryptophan were included as variable modifications. Precursor mass tolerance was set at 15 ppm and fragment ion mass tolerance was set at 0.9 Da for CID, 1.1 Da for ETD and 0.05 Da for HCD. All peptide identifications were combined using Scaffold and analyzed at a protein probability of 99% and a peptide probability of 95% as specified by the Peptide Prophet algorithm [19]. For the identification of disulfide linked peptides, the peak lists were also searched using the MassMatrix search engine [20] against the HisRS protein sequence. Identification of disulfide linkages was accepted at a minimum of 2 spectra (from any fragmentation method), with at least one of them having a minimum MassMatrix score of 15. All MassMatrix identified disulfide linked peptides are listed in Supplementary Fig. 1.

Construction of HisRS mutants

Mammalian expression plasmids containing the wild-type HisRS (HisRSwt) coding sequence or a deletion mutant that lacked the C-terminal cysteines at position 507 and 509 (ΔCIC) were generated using the previously described prokaryotic HisRS expression plasmid [21]. Primer 1 was used in combination with primer 2 or primer 3 (Supplementary Table 1) to generate PCR amplicons corresponding to HisRSwt or HisRSΔCIC, respectively. The 50 µl PCR reaction mixture contained 10 mM dNTPs (Promega, Madison), 10 pM forward primer, 10 pM reverse primer, 1x cloned Pfu reaction buffer, 0.5 U Pfu Hot start polymerase (Stratagene), and 10 ng plasmid DNA. PCR cycles were as following: denaturation at 95 °C for 1 min, primer annealing at 52 °C for 1 min, and extension at 72 °C for 2 min for 35 cycles. Both the PCR products and the pEGFP-C1 plasmid (Clontech) were digested with BglII (New England Biolabs (NEB), Ipswich) and NotI (HisRSwt and pEGFP-C1) or BglII and PstI (HisRS-ΔCIC and pEGFP-C1) and subsequently ligated in order to construct the pEGFP-HisRSwt or pEGFP-HisRS-ΔCIC, respectively. Cysteine-to-alanine mutants of HisRS were generated using the QuikChange site-directed mutagenesis approach (Stratagene). The

substituted cysteines are C83 (primers 4 and 5), C174 (primers 6 and 7), C191 (primers 8 and 9), C196 (primers 10 and 11), C224 (primers 12 and 13), C235 (primers 14 and 15), C379 (primers 16 and 17), and C455 (primers 18 and 19) (Supplementary Table 1). Also these amino acid substitution mutants were cloned into the pEGFP-C1 vector. The integrity of all constructs was verified by a Sanger-based sequencing method supported by Big Dye Terminator version 3 of Applied Biosystems.

Expression and in vitro oxidation of recombinant human HisRS

Recombinant HisRS protein production was induced by adding 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG, MP Biomedicals) to an overnight *E. coli* BL21(DE3)pLysS culture transformed with pET-3d-HisRS and the cells were cultured for an additional 4 hours [22]. Subsequently, bacteria were harvested by centrifugation and resuspended in buffer containing 50 mM Tris-HCl pH 7.4, 100 mM KCl, 1 mM EDTA, 0.1% NP-40, and 1 mM DTT. The bacteria were lysed by 10 min 30-second pulses in an ice-cold sonication waterbath (Bioruptor). Recombinant human HisRS was purified by two consecutive anion exchange chromatography steps. First, the cleared bacterial lysate, containing recombinant HisRS, was loaded on a DEAE sepharose (Pharmacia Biotech) column in buffer A (25 mM Tris/Bis, pH 6.8, 2 mM EDTA, 0.5 mM DTT) and bound material was eluted with a linear 0 – 1 M NaCl gradient in buffer A. The recombinant HisRS containing fractions were desalted using HiPrep™ 26/10 (Pharmacia Biotech) and loaded onto a Source Q15 column. The bound proteins were eluted by a linear 0 – 500 mM NaCl gradient in buffer A. The recombinant HisRS containing fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining, which showed that the purity was > 95%. Prior to the in vitro oxidation, the purified HisRS was desalted using a Thermo Zeba™ Desalt spin column, and subsequently subjected to the Fenton reaction in a 20 µl reaction mixture containing 0.1 mM $Fe(II)SO_4$ and 1.7 mM ascorbic acid for various time periods. The *in vitro* oxidation reaction was stopped by the addition of 10 mM EDTA.

Jurkat cell electroporation

Jurkat cells were transfected with the pEGFP-HisRS constructs using electroporation. Ten million Jurkat cells were washed with PBS and resuspended in 1 ml RPMI 1640 Glutamax™-I without any supplements prior to electroporation. Ten µg of DNA was transferred into 4 mm electroporation cuvettes (Cell Projects; EP-104) and mixed gently with 300 µl Jurkat cell suspension. The electroporation settings (960 µF and 250V) were optimized for Jurkat cells using Genepulser® II and resulted in a time constant of approximately 35 ms. After electroporation cell suspensions were gently resuspended in 10 ml RPMI 1640 Glutamax™-I medium supplemented with 1 mM sodium pyruvate, and 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH). Forty eight hours post transfection the cells were harvested by centrifugation at 1,000g for 5 min, and washed with PBS. Cell lysates were made as described above.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Prior to protein separation by SDS-PAGE, (non-) reducing sample buffer, containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, (5% β-mercaptoethanol,) and 0.005% Bromophenol Blue, was added to an equal volume of cell lysate. After heating at 95°C for 5 min, $0.75*10^6$ Jurkat cell equivalents were loaded per lane on 10% SDS-polyacrylamide gels and after electrophoresis transferred to nitrocellulose membranes by semi-dry western blotting. Protein transfer was checked by Ponceau S (Sigma-Aldrich) staining of the membrane. All further immunoblotting steps were performed under agitation at room temperature. Membranes were blocked with 3% non-fat dried milk in PBS in order to prevent non-specific binding. Patient sera, containing anti-HisRS antibodies, in a 2,500-fold dilution and monoclonal antibody in a 100-fold dilution in MPBST (3% non-fat dried milk, PBS, 0.05% Tween-20 (Fluka BioChemika)) were added and, after incubation for 1 hour at room temperature, bound antibodies were detected with IRDye-labeled goat anti-human antibodies or goat anti-mouse (LI-COR), 2,500-fold diluted in MPBST, followed by near-infrared detection and quantification with Odyssey 2.1 equipment and software.

In vitro aminoacylation

Normal and H_2O_2-treated (2 h) Jurkat cells were harvested by centrifugation, 1,000g for 5 minutes at room temperature, resuspended in 100 mM KCl, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, and 2.5 U/µl RNasin (Promega), 1 mM DTT and lysed by 5 30-second pulses in an ice-cold sonication waterbath (Bioruptor). The cell lysates were cleared by centrifugation at 21,000g for 30 min at 4 °C. The 50 µl aminoacylation reaction mixtures contained 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 20 mM Tris-HCl, pH 7.4, ^{14}C-histidine (Amersham), 2.5 U/µl RNasin and 10^7 Jurkat cell equivalents. After incubation for 1.5 hours at 37 °C, TRizol was used to

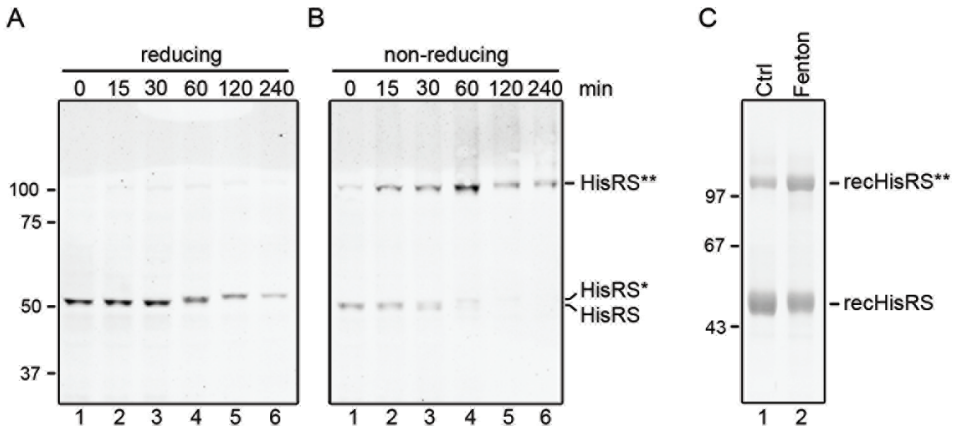


Fig. 1 ROS-induced covalent coupling of human HisRS dimers. Jurkat cells were exposed to 0.1% H_2O_2 for the indicated periods of time and cell lysates were prepared under reducing (A) or non-reducing conditions (B) and analyzed by immunoblotting. HisRS proteins (HisRS, HisRS* and HisRS**) were visualized with patient serum, containing anti-HisRS autoantibodies, and fluorescently labeled secondary antibodies. The positions of molecular weight markers are indicated on the left and those of (modified) HisRS on the right. (C) Purified recombinant human HisRS was oxidatively modified *in vitro* for 3 minutes using the Fenton reaction. Mock treated (Ctrl) and oxidized (Fenton) recombinant HisRS was separated by SDS-PAGE and proteins were detected by Coomassie Brilliant Blue staining. The positions of molecular weight markers are indicated on the left.

isolate total RNA, which was subsequently separated by electrophoresis in 6.5% polyacrylamide, 0.1 M NaOAc, pH 5.0, 7.5 M urea gels [23]. RNA was transferred to Hybond-N membranes (Amersham) and ^{14}C-labeled molecules were visualized by phosphorimaging. An antisense ^{32}P-labeled $tRNA^{His}$ probe was synthesized and used in northern blot hybridization as described previously [24]. Hybridized probes were visualized by phosphorimaging.

Results

Oxidative stress-induced covalent HisRS dimerization

Since oxidative stress may severely affect the redox status in the cytoplasm, HisRS from oxidatively stressed cells was analyzed by SDS-PAGE under both reducing and non-reducing conditions. Jurkat cells were treated with H_2O_2 for up to 4 hours, lysed either in the presence or absence of DTT and analyzed by SDS-PAGE after sample preparation either in the presence or in the absence of β-mercaptoethanol. After reducing sample preparation, mainly monomeric HisRS (54 kDa) was observed. During longer incubations an extra HisRS band (HisRS*) with decreased mobility could be seen (Fig. 1A), which is an oxidized form of HisRS [25]. In addition, a very weak signal was observed at approximately 110 kDa, which did not change as a result of the treatment with H_2O_2, although a similar slight migration shift was also observed for this polypeptide. The analysis under non-reducing conditions, however, showed a gradual decrease of the 54 kDa species of HisRS with a concomitant increase in the amount of a 110 kDa species (HisRS**, Fig. 1B). During the first two hours of treatment almost all 54 kDa molecules seem to be converted to the 110 kDa form. A comparison of the data in Fig. 1A and 1B shows that sample preparation in the presence of reducing agents almost completely converts the 110 kDa species to the 54 kDa form, which suggests that the 110 kDa form is generated by disulfide bond formation between HisRS and another polypeptide. Because HisRS is known to assemble into a homodimer and the apparent molecular mass is approximately twice that of monomeric HisRS, the 110 kDa species might represent a HisRS homodimer, in which both subunits are linked via at least one disulfide bond. The availability of purified recombinant human HisRS allowed us to investigate whether *in vitro* oxidation of the recombinant protein led to the same phenomenon. Indeed, the exposure of the recombinant HisRS to oxidative conditions generated by the Fenton reaction resulted in the conversion of the 54 kDa polypeptide to a 110 kDa form, when analyzed under non-reducing conditions (Fig. 1C). Taken together, these results indicate that oxidative conditions both in vivo and in vitro result in the formation of disulfide bonds between the two subunits of the HisRS homodimer.

Computational modeling of the human HisRS structure

To obtain an impression of the cysteines in the human HisRS protein that might be involved in disulfide bond formation, we generated a model for the three dimensional structure of the protein. Besides the N-terminal WHEP domain of the human HisRS, no

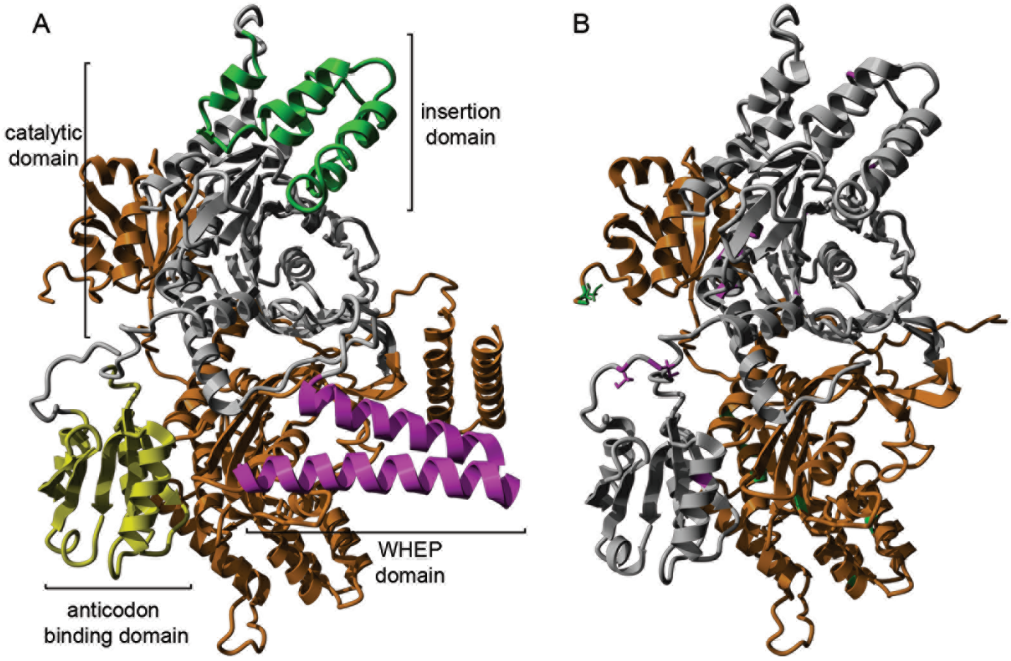
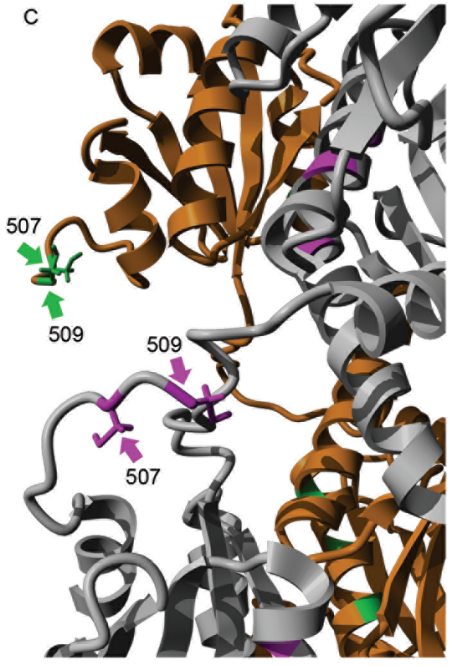


Fig. 2 Structural model of the human HisRS dimer. The quaternary structure of the human HisRS dimer was generated by computational modeling. (A) Subunit A is depicted in grey (catalytic domain), yellow (anticodon binding domain), green (insertion domain), and magenta (WHEP domain). Subunit B is shown completely in brown. (B) HisRS subunits A and B are shown in grey and brown, respectively, whereas all cysteines in both subunits are depicted in magenta and green, respectively. The WHEP domain was omitted from the model. (C) Enlarged view of the HisRS flexible C-terminal tail with cysteines (Cys507 and Cys509) involved in disulfide bond formation indicated in magenta and green.



structural data are available for this protein (Protein DataBank (PDB) accession number: 1X59), but several crystal structures of bacterial HisRS proteins have been published [26-29]. Recently, also the first crystal structures of eukaryotic HisRSs were published, namely that of *Trypanosoma brucei* and *Trypanosoma cruzi* HisRS [30]. Using the available HisRS structural data a model for the tertiary and quaternary structure of the human HisRS was built using YASARA software (www.yasara.org) The YASARA software selected pdb structure 1WU7, from *Thermoplasma acidophilum,* with an identity score of 25% as the structure that would fit best to the human HisRS structure (Supplementary Fig. 2). The result of computational modeling of the full-length dimeric human HisRS protein, based on the *Thermoplasma acidophilum* HisRS and the human WHEP domain structures, is presented in Figure 2A. The human WHEP domain had to be fit manually into the structure, because the software was unable to combine this part with the rest of the protein. Therefore, the positioning of this domain is speculative. As a consequence and in view of the absence of cysteines in this domain, we omitted this domain from the structures used for disulfide bond analysis (Fig. 2B). In the resulting dimeric structure all cysteines were highlighted in yellow (subunit A) or green (subunit B) (Fig. 2B). The distance

between most cysteine residues does not support the formation of disulfide bonds, except for two cysteine residues located close to the C-terminus of the polypeptide (Cys507 and Cys509). The C-terminal tail of HisRS seems to be rather unstructured and flexible, which may facilitate disulfide bond formation between these cysteine residues (Fig. 2C). In view of the putative structural flexibility of this region either one or two disulfide bonds between the two subunits can be envisaged.

Mapping of disulfide bonds in HisRS

To identify the cysteines involved in disulfide bond formation, we subjected the 110 kDa species of *in vitro* oxidized recombinant HisRS (Fig. 1B) to in-gel digestion using two different enzymes (trypsin and chymotrypsin) and analyzed the products by high resolution LC-MS/MS. Three different fragmentation techniques were applied to maximize coverage of the protein sequence. To facilitate the identification of residues involved in disulfide bonds, the protein was either only alkylated or reduced with DTT and subsequently alkylated prior to digestion. The sequence coverage of the human HisRS sequence is shown in Figure 3. When the protein was reduced prior to alkylation, the resulting sequences covered 96% of the protein, which included all cysteine residues except Cys83 and Cys191 (Fig. 3A). When the protein was not reduced before digestion, the total sequence coverage dropped to 81% and peptides containing Cys174, Cys196, Cys507 and Cys509 were not found, which may suggest that these residues are involved in disulfide bond formation in the dimer.

Because the Mascot search engine does not allow the identification of crosslinked peptides, we also analyzed all MS/MS spectra using the MassMatrix search engine [31], allowing the specific identification of both intermolecular and intramolecular disulfide containing peptides. This resulted in the identification of 4 unique disulfide containing fragments (identified with at least 2 spectra) of the recombinant HisRS dimer, which are listed in Table 1. One of these peptides corresponded to a disulfide bond between Cys83 and Cys235. However, three different peptides (corresponding to a total of 18 spectra) were identified that contained two disulfide bonds between Cys507 and Cys509, strongly supporting the notion that these two residues are involved in the interchain disulfide bonds covalently crosslinking the HisRS subunits in the dimer, as predicted by the structural model.

Interestingly, the double disulfide bridge between the cysteines close to the C-terminus of the two subunits suggests that two different configurations

A

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  1 MAERAALEELVKLQGERVRGLKQQKASAELIEEEVAKLLKLKAQLGPDESKQKFVLKTPK
 61 GTRDYSPRQMAVREKVFDVIIRCFKRHGAEVIDTPVFELKETLMGKYGEDSKLIYDLKDQ
121 GGELLSLRYDLTVPFARYLAMNKLTNIKRYHIAKVYRRDNPAMTRGRYREFYQCDFDIAG
181 NFDPMIPDAECLKIMCEILSSLQIGDFLVKVNDRRILDGMFAICGVSDSKFRTICSSVDK
241 LDKVSWEEVKNEMVGEKGLAPEVADRIGDYVQQHGGVSLVEQLLQDPKLSQNKQALEGLGD
301 LKLLFEYLTLFGIDDKISFDLSLARGLDYYTGVIYEAVLLQTPAQAGEEPLGVGSVAAGGR
361 YDGLVGMFDPKGRKVPCVGLSIGVERIFSIVEQRLEALEEKIRTTETQVLVASAQKKLLEE
421 RLKLVSELWDAGIKAELLYKKNPKLLNQLQYCEEAGIPLVAIIGEQELKDGVIKLRSVTSR
481 EEVDVRREDLVEEIKRRTGQPLCICE
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B

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  1 MAERAALEELVKLQGERVRGLKQQKASAELIEEEVAKLLKLKAQLGPDESKQKFVLKTPK
 61 GTRDYSPRQMAVREKVFDVIIRCFKRHGAEVIDTPVFELKETLMGKYGEDSKLIYDLKDQ
121 GGELLSLRYDLTVPFARYLAMNKLTNIKRYHIAKVYRRDNPAMTRGRYREFYQCDFDIAG
181 NFDPMIPDAECLKIMCEILSSLQIGDFLVKVNDRRILDGMFAICGVSDSKFRTICSSVDK
241 LDKVSWEEVKNEMVGEKGLAPEVADRIGDYVQQHGGVSLVEQLLQDPKLSQNKQALEGLGD
301 LKLLFEYLTLFGIDDKISFDLSLARGLDYYTGVIYEAVLLQTPAQAGEEPLGVGSVAAGGR
361 YDGLVGMFDPKGRKVPCVGLSIGVERIFSIVEQRLEALEEKIRTTETQVLVASAQKKLLEE
421 RLKLVSELWDAGIKAELLYKKNPKLLNQLQYCEEAGIPLVAIIGEQELKDGVIKLRSVTSR
481 EEVDVRREDLVEEIKRRTGQPLCICE
```

Fig. 3 Sequence coverage of HisRS by LC-MS/MS data. Sequences that were found in peptides identified by mass spectrometric analysis of the (oxidatively modified) recombinant HisRS are marked by gray shading, both for the data obtained with the reduced protein (panel A) and for data obtained with the non-reduced protein (panel B). All cysteine residues in the sequence are boxed.

of the linkage are possible, namely a parallel (Cys507-Cys507 and Cys509-Cys509) and an anti-parallel linkage (twice Cys507-Cys509). Because the peptides resulting from these two configurations would have significantly different structures, we hypothesized that these might be separable by C18 reversed phase column chromatography. In addition, specific reporter ions in the resulting MS/MS spectra resulting from at least two fragmentation events in the peptide might reveal the relative orientation of C-terminal elements in the covalent dimer. In Figure 4A, extracted ion chromatograms of the precursor mass of one of these peptides (MH^+ 1663.73; m/z 832.37^{2+}) are shown for the tryptic digests of HisRS. These chromatograms show two distinct peaks in the non-reduced (-DTT) sample, which are both abolished upon reduction (+DTT), suggesting the presence of both orientations of the two bonds in the HisRS sample. To discriminate between the two configurations, we manually annotated the HCD generated MS/MS spectra of this disulfide containing peptide (Fig. 4B), and used specific reporter fragment ions to show that the first eluting peak originated from the parallel configuration and the second peak from the anti-parallel configuration (Fig. 4A, lower panels) of the two peptide chains.

Confirmation of intermolecular disulfide bond formation by C-terminal cysteines in vivo

To confirm the involvement of the two cysteines close to the C-terminus of HisRS in intermolecular disulfide bond formation and to demonstrate that this also occurs *in vivo*, we investigated the effects of

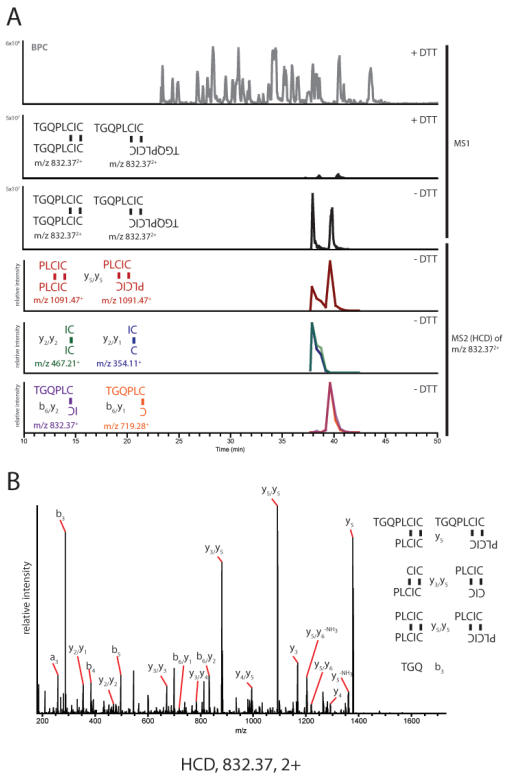

Fig. 4 Detailed LC-MS/MS analysis of intermolecularly crosslinked C-terminal peptide. (A) LC-MS/MS chromatograms of the tryptic digest of reduced (top 2 traces) and non-reduced HisRS (bottom 4 traces). The top trace (gray) shows the base peak chromatogram (BPC) with all observed peptide peaks. In black, the extracted ion chromatogram trace of the homodimer of peptide TGQPLCIC (m/z 832.37) is shown for the reduced (+DTT) and non-reduced (-DTT) HisRS protein, showing the presence of two isoforms of this peptide in the non-reduced HisRS. The hypothesized structure of the two isoforms is inserted in the left part of the chromatogram. The colored traces show the presence of specific reporter fragment ions in the HCD fragmentation spectra of the peptide with m/z 832.37. In red, the trace of the y5/y5 fragment is shown, which is present in both isoforms of the peptide. The green (y2/y2) and blue (y2/y1) traces show fragments specific for the first eluting isoform of the peptide, and the purple (b6/y2) and orange (b6/y1) trace correspond to fragments that can only be generated by fragmentation of the second isoform. (B) An HCD MS2 spectrum of the peptide with m/z 832.37, averaged over both isoforms, with all identified fragment ions marked. Fragments generated from a single cleavage are indicated by the normal nomenclature and fragments generated from double cleavage are indicated with the identifier of both resulting, disulfide linked, fragments. The structure of the four most intense ions is depicted to the right of the spectrum.

amino acid substitutions on the ability of ectopically expressed HisRS to form covalent dimers upon the induction of oxidative stress in Jurkat cells. Jurkat cells transfected with constructs encoding the EGFP-tagged wild-type or mutant HisRS were treated with 0.1% H_2O_2 for 1 hour, to generate the oxidative conditions that stimulate disulfide bond formation in HisRS. Subsequently, cell lysates were analyzed by immunoblotting using antibodies to HisRS and EGFP. The EGFP-HisRS fusion protein can dimerize both with another EGFP-HisRS molecule or with endogenous HisRS, resulting in covalent dimers with distinct electrophoretic mobilities. The relative amounts of these dimers will depend on the relative concentrations of these molecules in the transfected cells. The major anti-EGFP reactive dimer band most likely represents the EGFP-HisRS homodimer, because the concentration of newly synthesized EGFP-HisRS (in transfected cells) will be significantly higher than that of newly synthesized endogenous HisRS. Therefore, the chance for an EGFP-HisRS molecule to encounter another EGFP-HisRS molecule will be much higher compared to a non-tagged HisRS partner. The results showed that all cysteine substitution mutants were able to form covalent HisRS dimers, with the exception of the C-terminal deletion mutant (ΔCIC), which lacks the 3 most C-terminal residues, including Cys507 and Cys509 (Fig. 5). These data strongly suggest that intermolecular bonds between the HisRS subunits, formed under oxidative conditions, are only formed between the C-terminal cysteines and that no other disulfide bonds contribute to the covalent linkage between the subunits of the dimer.

Oxidative modifications of HisRS do not abrogate its tRNA aminoacylation activity

To investigate whether the oxidative modifications, including the formation of disulfide bonds, of HisRS affect its capacity to catalyze the aminoacylation of histidyl-tRNA, in vitro aminoacylation reactions were performed using ^{14}C-histidine and lysates from either normal or oxidatively stressed Jurkat cells. The conjugation of radiolabeled histidine to $tRNA^{His}$ was monitored by autoradiography. RNA was isolated

Table 1. List of peptides containing disulfide bonds in dimerized recombinant HisRS.

Peptide sequence[a]	MH+	AA involved	Intra/inter chain	# peptides
TIC($)SSVDKLDK + C($)FKR	1758.88	C83, C235	Inter or Intra	2
RTGQPLC($)IC($) + RTGQPLC($)IC($)	1975.93	C507, C509	Inter	4
RTGQPLC($)IC($) + TGQPLC($)IC($)	1819.83	C507, C509	Inter	7
TGQPLC($)IC($) + TGQPLC($)IC($)	1663.73	C507, C509	Inter	7

[a] Dollar signs indicate the position(s) of crosslinking

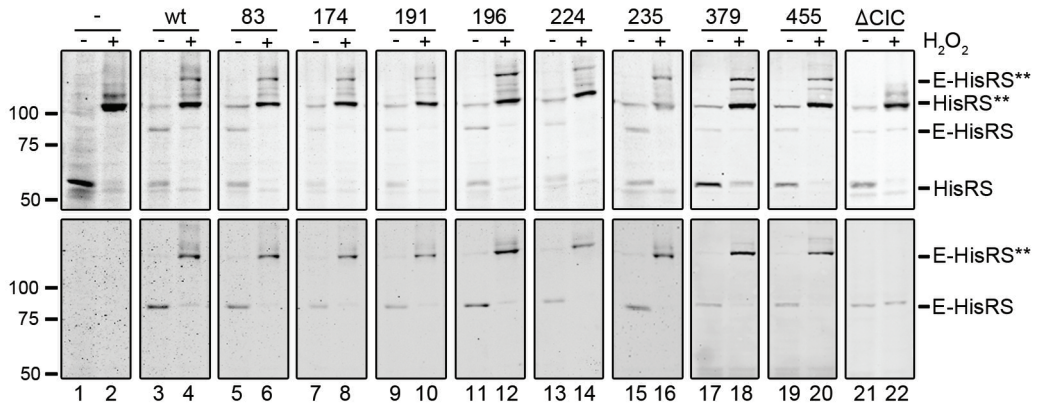

Fig. 5 Effect of cysteine substitutions on disulfide bond formation in HisRS dimers. Extracts of Jurkat cells, transfected with constructs encoding EGFP-tagged HisRS mutants and treated with 0.1% H_2O_2 for 1 hour (+ lanes) or untreated (- lanes), were analyzed for the presence of covalent HisRS dimers by non-reducing SDS-PAGE and immunoblotting using anti-HisRS and anti-EGFP antibodies. The upper panels show the signals obtained with anti-HisRS autoantibodies. Monomeric HisRS (HisRS) and crosslinked HisRS (HisRS**) signals were detected at 54 kDa and 110 kDa, respectively. In addition, signals that correspond to (crosslinked) EGFP-tagged HisRS were detected at approximately 80 kDa (E-HisRS), and 160 kDa (E-HisRS**). The lower panel shows the results obtained with a monoclonal anti-EGFP antibody, which confirms the positions of the EGFP-tagged (mutant) HisRS molecules. Jurkat cells were transfected with constructs encoding EGFP-tagged: wt: wild type HisRS; 83: HisRS-C83A; 174: HisRS-C174A, 191: HisRS-C191A; 196: HisRS-C196A; 224: HisRS-C224A; 235: HisRS-C235A; 379: HisRS-C379A; 455: HisRS-C455A; ΔCIC: HisRS-ΔCIC; or mock transfected (−). Cells were harvested 48 hours post-transfection.

from aminoacylation reaction mixtures, separated by denaturating gel electrophoresis and transferred to Hybond-N membranes. The aminoacylated $tRNA^{His}$ signals were visualized via direct phosphorimaging, whereas ^{32}P-labeled antisense probes were used to detect total (histidyl-)$tRNA^{His}$ by northern blot hybridization (Fig. 6). The results of the northern blot showed that $tRNA^{His}$ levels were comparable between the individual samples. Surprisingly, the aminoacylation of $tRNA^{His}$ was not diminished in lysates from the H_2O_2-treated cells; the signals obtained were reproducibly stronger (approximately 1.6-fold, as quantified by phosphorimaging) than those in the untreated cells. (Fig. 6).

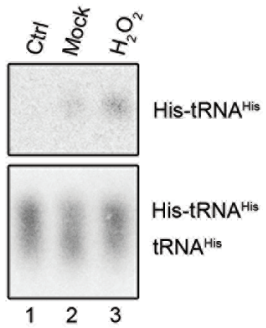

Fig. 6 HisRS aminoacylation activity. Jurkat cells were mock-treated or H_2O_2-treated, prior to the aminoacylation assay. The control aminoacylation assay samples (Ctrl) were supplemented with a 1,000-fold excess of unlabeled-histidine. The conjugated ^{14}C-histidyl-$tRNA^{His}$ was analyzed via RNA isolation followed by denaturing gel electrophoresis, after which radiolabeled RNAs were visualized by phosphorimaging. Total $tRNA^{His}$ was detected by northern blot hybridization using a ^{32}P-labeled antisense probe.

Discussion

The formation of intermolecular or intramolecular disulfide bridges in hetero- or homo-multimeric complexes might fine-tune protein activity or regulate protein rigidness that is needed for specific conformational structures. In this study we identified oxidative stress induced disulfide bridges in homodimeric HisRS.

The aaRSs fulfill an essential role in protein synthesis, and mischarging of tRNAs and/or dysregulation of the aminoacylation activity of aaRSs can severely impair the production of proteins. Aminoacylation of tRNAs thus can be a rate-limiting step in protein synthesis and optimal aaRS activity has to be ensured for proper translation. We previously showed that HisRS is oxidatively modified upon H_2O_2-induced stress which resulted in several oxidized amino acids in endogenous as well as recombinant HisRS [25]. Protein oxidation is often associated with the inhibition of protein activity and/or function and can result in degradation by the proteosome. However, an increasing number of proteins functioning in different cellular processes have been reported to be regulated in a redox-dependent manner [32-35]. The redox-regulated protein activity is associated with the oxidation and/or disulfide bond formation of cysteine residues in the emerging oxidative environment. A variable intracellular redox-state might be involved in the regulation of several different cellular processes

Supplementary Table 1. Primer sequences.

Primer	Sequence (5' - 3')
1	GGGGAGATCTATGGCAGAGCGTGCGGCG[a]
2	GGGGGCGGCCGCTCAGCAGATGCAGAGG[a]
3	GGGGCTGCAGTCAGAGGGGCTGGCCTGTTC[a]
4	TAATCATCCGTGCCTTCAAGCGCC
5	GGCGCTTGAAGGCACGGATGATTA
6	GAATTCTACCAGGCCGATTTTGACATTG
7	CAATGTCAAAATCGGCCTGGTAGAATTC
8	CTGATGCAGAGGCCCTGAAGATCATG
9	CATGATCTTCAGGGCCTCTGCATCAG
10	CTGAAGATCATGGCCGAGATCCTGAGTTC
11	GAACTCAGGATCTCGGCCATGATCTTCAG
12	GATGTTTGCTATCGCCGGTGTTTCTGAC
13	GTCAGAAACACCGGCGATAGCAAACATC
14	GTTCCGTACCATCGCCTCCTCAGTAGACAAG
15	CTTGTCTACTGAGGAGGCGATGGTACGGAAC
16	GCGCAAGGTGCCAGCCGTGGGGCTCAGCATTG
17	CAATGCTGAGCCCCACGGCTGGCACCTTGCGC
18	CCAGTTACAGTACGCCGAGGAGGCAGGCATC
19	GATGCCTGCCTCCTCGGCGTACTGTAACTGG

[a] BglII, NotI, and PstI restriction sites are underlined in primers 1, 2, and 3, respectively

as a response to specific (patho-)physiological signals. Protein synthesis has been suggested to be sensitive to oxidative stress [36,37]. The data obtained in this study suggest that HisRS activity might be modulated by redox-fluctuations as well. To explain this at the molecular level, we have generated the first structural model for the human HisRS (Fig. 2) based upon an existing prokaryotic HisRS structure, which gives insight into the oxidative-stress induced disulfide bridge formation.

Most of the cysteines in the human HisRS model are located at distances that are not suitable for disulfide bond formation. Only two cysteines near the C-terminal end of HisRS Cys507 and Cys509), appeared to be potentially involved in disulfide bond formation. The combined results of mass spectrometry and MassMatrix analysis suggested that C83, C174, C196, C235, C507, and C509 might be involved in crosslinking HisRS dimers via disulfide bonds. The HisRS derived peptides containing C174, C196, C507, and C509 were not detectable in mass spectra obtained with non-reduced samples, in contrast to those obtained with reduced HisRS samples. This might have been caused by a shift in the m/z values of the respective peptides, e.g. as a result of disulfide formation. Alternatively, oxidative conditions might affect redox-sensitive protease cleavage sites, although this possibility is not very likely, because two proteases with distinct specificities were used. The MassMatrix search engine suggested the potential involvement of residues C83 and C235 in disulfide bond formation, although the probability was low (Table 1). Cysteine-to-alanine mutagenesis of these residues showed no effect on the covalent HisRS dimerization, indicating that these cysteines are not involved in intermolecular disulfide bonds. However, an intramolecular crosslink between C83 and C235 might still be formed, although this does not seem to be very likely based upon their positions in the structural model for HisRS. In contrast, the formation of two intermolecular disulfide bonds by C507 and C509, as predicted by the structural model, was supported by both the MassMatrix and mutagenesis results. A detailed analysis of the LC-MS/MS data revealed that these residues can lead to two distinct crosslinked configurations, one in which C507 and C509 of one subunit are coupled to the same residues of the other subunit, and a second in which C507 of one subunit is coupled to C509 of the other and vice versa. The occurrence of both configurations is consistent with the putative flexible nature of the C-terminal part of HisRS as predicted by the structural model.

The results of the experiments in which the aminoacylation of $tRNA^{His}$ was investigated in lysates from either mock-treated or oxidatively stressed cells indicate that the oxidative modification of HisRS does not abrogate its enzymatic activity. Instead, a slight increase of $tRNA^{His}$ aminoacylation was reproducibly observed. In contrast to these observations, pyrophosphate exchange and aminoacylation activity were reported to be abolished by the modification of sulfhydryl groups of a prokaryotic HisRS. It was suggested that these modifications induced structural changes that affect aminoacyl activation instead of being directly involved in substrate binding of catalytic processes [38,39]. Why would HisRS become more active as a result of these modifications? Francklyn and Arnez comprehensively described the structural domains of HisRS, as well as substrate binding interactions and the mechanism of tRNA aminoacylation [40]. In addition, they developed a model in which conformational changes in the dimeric HisRS are important for proper tRNA aminoacylation. An obvious effect of the formation of disulfide bonds between the two subunits of the dimer is stabilization of the dimer. It is well established that dimerization of HisRS is required for its enzymatic acitivity, and a large fraction of the HisRS pool appears to exist as dimers in HeLa cells [41]. If both subunits of the dimer dissociate as a result of the reaction they catalyze, e.g. to facilitate the release of the charged tRNA, then the dimer may be more easily restored when both

A

MassMatrix identified disulfide linked peptides from non-reduced HisRS

Index	scan#	charge	score	pp	pp_2	pp_{tag}	m/z	MW(obs)	MW	delta	miss	Unique	sequence + modifications [start:end]
1205	245	+4	11	6.6	3.2	2.6	401.4511	1602.7827	1602.7866	-0.0039	1	×	T(C($1)SSVDKLDK C($1)FK [A233:243 A83:85]
1319	171	+4	18	9.9	6.7	7.4	440.4774	1758.8876	1758.8877	-0.0001	2	×	T(C($1)SSVDKLDK C($1)FKR
1320	170	+5	14	9.8	4.1	5.1	352.5834	1758.8878	1758.8877	0.0001	2	×	T(C($1)SSVDKLDK C($1)FKR [A233:243 A83:86]
1326	822	+2	13	4.9	4.8	5.8	910.4189	1819.8305	1819.8322	-0.0017	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x)
1327	754	+3	28	5.8	8.2	6.0	607.2819	1819.8310	1819.8322	-0.0011	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x)
1328	840	+3	19	3.4	6.1	2.0	607.2823	1819.8323	1819.8322	0.0001	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x)
1329	886	+3	23	3.8	7.6	2.7	607.2826	1819.8332	1819.8322	0.0010	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x)
1330	731	+3	26	4.9	8.1	3.9	607.2828	1819.8338	1819.8322	0.0016	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x)
1331	819	+3	29	5.8	8.2	4.6	607.2828	1819.8338	1819.8322	0.0016	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x) [A501:509 B502:509]
165	964	+2	16	6.3	8.2	2.6	416.6870	832.3668	832.3692	-0.0024	0	×	TGQPLC($1)IC($1) [A502:509]
1256	952	+3	16	2.4	5.6	5.8	555.2485	1663.7309	1663.7311	-0.0002	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x)
1259	994	+2	10	1.8	4.8	6.6	832.3697	1663.7321	1663.7311	0.0010	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x)
1265	889	+2	10	2.1	5.0	6.6	832.3704	1663.7334	1663.7311	0.0024	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x)
1266	931	+2	12	1.8	5.2	5.0	832.3706	1663.7339	1663.7311	0.0029	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x) [A502:509 B502:509]
1296	400	+6	22	7.1	6.0	3.0	462.2340	2768.3675	2768.3892	-0.0217	1	√	VEEIKRTGQPLC($1)IC AIC($1)GVSDSKF [A495:509 A222:231

CID

Index	scan#	charge	score	pp	pp_2	pp_{tag}	m/z	MW(obs)	MW	delta	miss	Unique	sequence + modifications [start:end]
1372	614	+3	14	1.8	7.1	5.0	659.3151	1975.9306	1975.9333	-0.0027	2	√	RTGQPLC($x)IC($x) RTGQPLC($x)IC($x)
1373	612	+4	13	2.5	7.3	4.2	494.7382	1975.9308	1975.9333	-0.0025	2	√	RTGQPLC($x)IC($x) RTGQPLC($x)IC($x)
1374	707	+4	10	1.9	6.1	1.6	494.7394	1975.9357	1975.9333	0.0024	2	√	RTGQPLC($x)IC($x) RTGQPLC($x)IC($x)
1375	712	+3	18	1.5	8.3	6.8	659.3172	1975.9370	1975.9333	0.0038	2	√	RTGQPLC($x)IC($x) RTGQPLC($x)IC($x) [A501:509 B501:509]
1256	952	+3	12	5.4	9.6	14.6	555.2485	1663.7309	1663.7311	-0.0002	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x)
1260	911	+2	10	2.3	5.6	2.2	832.3699	1663.7325	1663.7311	0.0014	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x)
1266	931	+2	10	0.9	5.9	2.2	832.3706	1663.7339	1663.7311	0.0029	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x) [A502:509 B502:509]

ETD

Index	scan#	charge	score	pp	pp_2	pp_{tag}	m/z	MW(obs)	MW	delta	miss	Unique	sequence + modifications [start:end]
1239	802	+2	15	32.5	18.8	4.8	910.4189	1819.8305	1819.8322	-0.0017	1	√	RTGQPLC($x)IC($x) TGQPLC($x)IC($x) [A501:509 B502:509

HCD

B

MassMatrix identified disulfide linked peptides from reduced HisRS

Index	scan#	charge	score	pp	pp_2	pp_{tag}	m/z	MW(obs)	MW	delta	miss	Unique	sequence + modifications [start:end]
1389	862	+3	41	5.3	8.9	8.1	645.9677	1935.8884	1935.8908	-0.0024	1	×	RTGQPLCIC($1) TGQPLCIC($1) + CAMC(7)+ CAMC(17) [A501:509 B502:509]
1326	843	+3	22	2.4	7.5	3.0	607.2827	1819.8334	1819.8322	0.0012	1	×	RTGQPLC($x)IC($x) TGQPLC($x)IC($x) [A501:509 B502:509]
1238	993	+2	10	1.8	5.0	5.0	832.3694	1663.7316	1663.7311	0.0005	0	√	TGQPLC($x)IC($x) TGQPLC($x)IC($x) [A502:509 B502:509]
1310	1007	+2	28	2.0	6.5	13.0	890.3972	1779.7870	1779.7896	-0.0026	0	√	TGQPLCIC($1) TGQPLCIC($1) + CAMC(6)+ CAMC(16) [A502:509 B502:509]
76	336	+2	11	3.9	9.7	3.4	394.1341	787.2609	787.2605	0.0004	0	√	CIC($1) CIC($1) + CAMC(1)+ CAMC(6) [A507:509 B507:509]

CID

Index	scan#	charge	score	pp	pp_2	pp_{tag}	m/z	MW(obs)	MW	delta	miss	Unique	sequence + modifications [start:end]
1348	855	+3	12	23.5	22.1	13.3	645.9677	1935.8884	1935.8908	-0.0024	1	√	RTGQPLCIC($1) TGQPLCIC($1) + CAMC(7)+ CAMC(17) [A501:509 B502:509

HCD

Supplementary Fig. 1 Disulfide linked peptides identified by MassMatrix.
The MS/MS spectra of non-reduced and reduced HisRS samples were analyzed for crosslinked peptides using the MassMatrix search engine. (**A**) Four intra- and/or intermolecular peptide crosslinks with a MassMatrix score of at least 15 in either of the fragmentation methods (trypsin and chymotrypsin) and at least 2 spectra matches were detected in peak lists of the non-reduced HisRS sample. (**B**) No intra- or intermolecular peptide crosslinks that meet these criteria were found in the reduced HisRS spectra.

subunits remain covalently attached to each other via the flexible C-terminal tail. Alternatively, the disulfide bonds may affect the induced-fit mechanisms involved in structuring the histidine-binding pocket. Moreover, it should be stressed that the oxidative conditions also induce the oxidation of 4 methionine and 1 tryptophan residue of HisRS (Van Dooren *et al.* 2011), as well as modifications of many other cellular components. Obviously, also these changes may affect the aminoacylation activity in cell lysates. Further studies will be required to shed more light on this issue.

In conclusion, the generation of the first structural model of human HisRS helped to explain the formation of disulfide bonds between the subunits of the HisRS homodimer, which are generated under oxidative conditions. Two cysteine residues in the flexible C-terminal region are involved in the covalent linkage between these subunits, which may, in combination with other oxidative modifications, slightly enhance the catalytic activity of HisRS in $tRNA^{His}$ aminoacylation.

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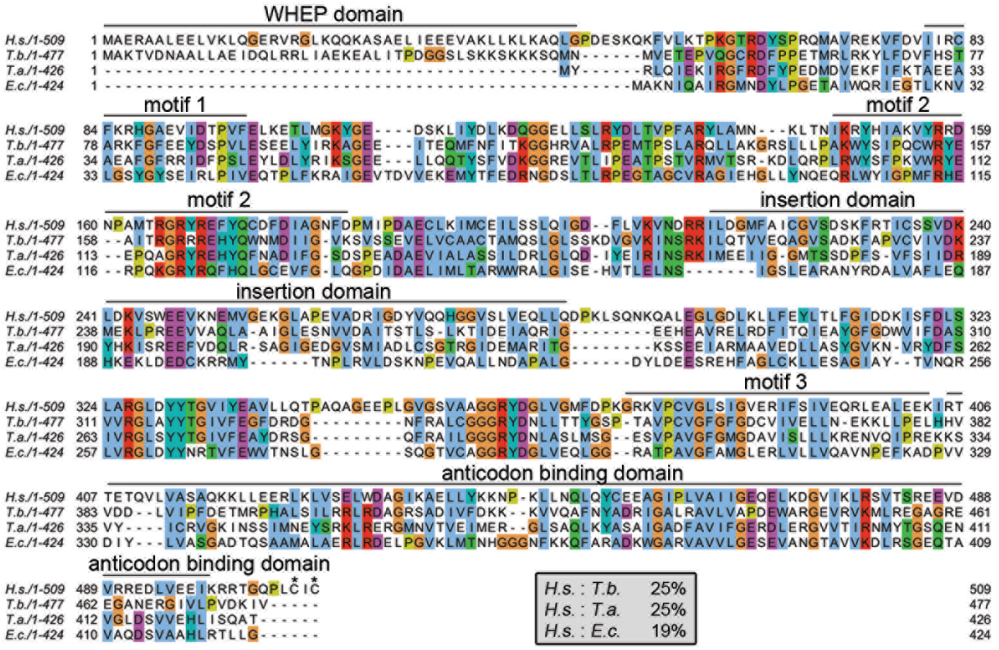

Supplementary Fig. 2 Multiple sequence alignment of HisRS.
ClustalW was used to align the sequences of HisRS from H.s.: *Homo sapiens*, T.b.: *Trypanosoma brucei*, T.a.: *Thermoplasma acidophilum*, and E.c.: *Escherichia coli* (K12). Pairwise and multiple alignment settings were set at default. Pairwise sequence alignment identity scores of the human HisRS compared to the other HisRSs are depicted in the grey box. Identical and similar amino acids were highlighted by Jalview v.2.4. The amino acids that form the WHEP domain, insertion domain, catalytic domain motifs, and anticodon binding domain are indicated with a horizontal black line. Asterisk: cysteine residue involved in disulfide bond formation.

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Chapter 6

SIN-1-induced reactive oxygen/nitrogen species lead to covalent linkages between subunits of the homodimeric histidyl-tRNA synthetase

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Abstract

The histidyl-tRNA synthetase, also known as the Jo-1 autoantigen, is one of the main targets of the immune system in myositis, which is characterized by chronic inflammation of the skeletal muscles. Autoantibodies are found in up to 80% of polymyositis/dermatomyositis patients and antibodies to HisRS are the most prevalent myositis-specific autoantibodies. Post-translational modifications have been suggested to initiate the loss of immunological tolerance and result in the production of autoantibodies in a number of autoimmune diseases. Since infiltrating lymphocytes and necrotic loci are found in skeletal muscle tissue of most myositis patients, free radicals associated with oxidative or nitrosative stress have been implicated as important modifying agents. To investigate whether HisRS is modified in oxidatively/nitrosatively stressed cells, Jurkat cells were subjected to agents producing intracellular reactive oxygen/nitrogen species (ROS/RNS). The HisRS polypeptides, which are known to assemble into homodimers, appeared to be crosslinked under these conditions. The covalent homodimerization was resistant to reducing agents indicating that disulfide bonds were not involved. Superoxide and peroxynitrite scavengers inhibited the formation the covalent bonds substantiating the involvement of ROS/RNS. The results of mutagenesis experiments showed that the covalent homodimerization of HisRS involves cysteines in the C-terminal tail of the protein. Neither cell death nor alterations in the tRNA aminoacylation activity of HisRS were detected in SIN-1-treated Jurkat cells, although cell growth and translation were inhibited. Autoantibodies in myositis patient sera that specifically target the crosslinked HisRS dimer were not detected.

Introduction

Myositis or idiopathic inflammatory myopathies (IIM) can be subdivided into 3 main disorders, polymyositis (PM), dermatomyositis (DM) and inclusion-body myositis (IBM), all with their own distinct serological and histological features [1,2]. In up to 80% of PM and DM patients myositis-associated autoantibodies (MAA) or myositis-specific autoantibodies (MSA) can be found, directed against a variety of ubiquitously expressed cellular components [3,4]. Myositis-specific autoantibodies against the main autoantigen, histidyl-tRNA synthetase (HisRS; anti-Jo-1 antibodies) are found in 20% to 30% of PM/DM patients. Although the exact etiology of myositis remains unknown, the large number of inflammatory cells and the presence of autoantibodies found in these patients suggest that autoimmune processes are involved. The generation of neo-epitopes as a result of modifications of self-proteins has been suggested to initiate an immune response and autoimmunity. In addition, multiple factors including environmental, genetic, and hormonal factors and defects in the clearance of cell remnants may contribute to the loss of immunological tolerance.

The levels of reactive oxygen and reactive nitrogen species (ROS/RNS) are increased in autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [5]. The direct detection of ROS *in vivo* is difficult due to their high reactivity with biomolecules such as nucleic acids, lipids and proteins. Moreover, ROS and RNS contain different reactive moieties that result in distinct protein modifications that affect protein activity, folding, processing, and antigenicity. Such activities may facilitate the measurement of ROS/RNS end products and the determination of intracellular levels of radicals. Studies on synovial fluid (SF), tissue and sera from RA patients showed oxidized low-density-lipoproteins, elevated levels of protein carbonylation and increased recognition of 3-nitrotyrosine by IgGs isolated from SF [6-8]. In SLE anti-nitrotyrosine antibodies have been reported that crossreact with single-stranded DNA, and in experimental animals elevated immunogenicity of RNS-modified DNA compared to native DNA was observed, as well as increased serum levels of 3-nitrotyrosine and protein carbonylation [9-11]. These studies suggest the generation of neo-epitopes by the exposure to ROS/RNS, through post-translational modifications of biomolecules, which are able to stimulate autoreactive T-cells and B-cells. Interestingly, De Paepe and colleagues reported elevated levels of inducible nitric oxide synthase (iNOS) expression in endomysial infiltrates of PM patients, which suggests the increased production of nitric oxide (NO) and other reactive nitrogen intermediates in the myositis muscle [12]. In addition, skeletal muscle tissue is known to produce ROS/RNS in rest, and upon exercise or tissue damage the ROS/RNS levels increase significantly [13]. The elevated levels of ROS/RNS may damage the surrounding tissues and modify extracellular and/or intracellular proteins. The production of equimolar amounts of $NO^{\cdot}$ and $O_2^{\cdot-}$ (superoxide anion) may lead to the generation of readily diffusible peroxynitrite ($ONOO^-$) molecules in the muscle tissue microenvironment. Peroxynitrite has been associated with protein modifications which are found in cytotoxicity and several autoimmune diseases.

In this study we investigate the effects of ROS/RNS on the major myositis-specific autoantigen, HisRS, using the metabolite SIN-1 to stimulate the formation of intracellular $ONOO^-$. We observed covalent crosslinking of the HisRS protein in SIN-1 treated Jurkat cells. Insight into the chemical nature of the covalent linkage is provided and the possibility that this altered structure plays a role in the recognition by myositis antibodies is investigated.

Materials and methods

Cell culture, electroporation and metabolic labeling

Jurkat T-lymphoma cells were cultured in RPMI 1640 Glutamax™-I medium supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin (Gibco) and 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH). Cells were maintained at a maximum density of 10^6 cells/ml in a humidified incubator at 37 °C with 5% CO_2. Jurkat cells were transfected with plasmid DNA by electroporation. Ten million cells per transfection were washed with PBS and resuspended in 1 ml culture medium without supplements. Subsequently, 10 µg DNA was gently mixed with 300 µl Jurkat cell suspension in 4 mm electroporation cuvettes (Cell Projects). The electroporation settings (960 µF and 250V) were optimized for Jurkat cells using Genepulser® II and resulted in a time constant of approximately 35 ms. After electroporation cell suspensions were gently mixed and resuspended in 10 ml RPMI 1640 Glutamax™-I medium supplemented with 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH). Forty-eight hours post-transfection the cells were harvested by centrifugation at 1,000xg for 5 min, and washed with PBS. For metabolic labeling logarithmically growing Jurkat cells were exposed for 6 hours to 0.4

Table 1. Primer sequences

Primers	Sequence (5' - 3')
1	GTGCTCAAAACCCCCGCCGGCACAAGAGAC
2	GTCTCTTGTGCCGGCGGGGGTTTTGAGCAC
3	AAGGGCACAAGAGCCTATAGTCCCCGG
4	CCGGGGACTATAGGCTCTTGTGCCCTT
5	GATACACCTGTATTTGCCCTAAAGGAAACACTGATGG
6	CCATCAGTGTTTCCTTTAGGGCAAATACAGGTGTATC
7	GTATGGGGAAGACTCCGCCCTTATCTATGACCTG
8	CAGGTCATAGATAAGGGCGGAGTCTTCCCCATAC
9	GAAGACTCCAAGCTTATCGCTGACCTGAAGGACCAGGGC
10	GCCCTGGTCCTTCAGGTCAGCGATAAGCTTGGAGTCTTC
11	GCGCAAGGTGCCAGCCGTGGGGCTCAGCATTG
12	CAATGCTGAGCCCCACGGCTGGCACCTTGCGC
13	CCAGTTACAGTACGCCGAGGAGGCAGGCATC
14	GATGCCTGCCTCCTCGGCGTACTGTAACTGG
15	GGGGAGATCTATGGCAGAGCGTGCGGCG
16	GGGGCTGCAGTCAGAGGGGCTGGCCTGTTC

mM SIN-1 or buffer as a control, after which 9 µM (concentration; 11 mCi/ml, specific activity; >1000 Ci/mmol) [^{35}S]methionine (PerkinElmer) was added. Cells were cultured for another 4 hours and at the indicated time points, 2*10^6 cells were collected. Cell lysates were prepared according to the procedures described below. For cell growth experiments Jurkat cells were mock- or SIN-1 (0.4 mM) treated and every 24 hours another dose of 0.4 mM SIN-1 was added. Cell numbers were determined in triplicate with a Coulter A^c•T 8 (Coulter Series Analyzer, Miami, US) at 0, 8, 24, 48, and 72 hours after adding the first dose of SIN-1.

Induction of ROS/RNS stress and cell lysate preparation

The cells were exposed to either 0.4 mM SIN-1 (Enzo Life Sciences; Plymouth Meeting, PA) for the indicated time periods or incubated with various SIN-1 concentrations for 8 hours at 37 °C. Cell suspensions were harvested by centrifugation at 1,000xg for 5 min, and washed with PBS. Cell pellets were resuspended in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 0.1% Nonidet-P40 (NP-40), and 1 mM DTT and lysed by sonication on ice. Insoluble material was removed by centrifugation at 21,000xg for 30 min at 4 °C and supernatants were used immediately.

Gelelectrophoresis and immunoblotting

Prior to protein separation by SDS-PAGE, reducing sample buffer, containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercapto-ethanol, and 0.005% Bromophenol Blue, was added to an equal volume of cell lysate. After heating for 5 min at 95 °C, 0.75*10^6 Jurkat cell equivalents per lane were separated by electrophoresis in 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes by semi-dry western blotting. Protein transfer was checked by Ponceau S (Sigma-Aldrich) staining. All further immunoblotting steps were performed under agitation at room temperature. Membranes were blocked with 3% non-fat dried milk in PBS. Patient serum in a 2,500-fold dilution and monoclonal antibody in a 100-fold dilution in PBS containing 0.05% Tween-20 (Fluka BioChemika; MPBST) was added and bound antibodies were detected with IRDye-labeled goat anti-human antibodies or goat anti-mouse antibodies (LI-COR), respectively, 2,500-fold diluted in MPBST, followed by near-infrared detection and quantification with Odyssey 2.1 software (LI-COR).

Site-directed mutagenesis

The coding sequence of HisRS was isolated from a previously described prokaryotic HisRS expression plasmid by PCR (50 µl reaction mixture containing 10 mM dNTPs, 10 pM forward primer, 10 pM reverse primer, 1x cloned Pfu reaction buffer, 0.5 U Pfu Hot start polymerase (Stratagene), and 10 ng plasmid DNA) [14]. PCR cycles were as follows: denaturation at 95 °C for 1 min, primer annealing at 52 °C for 1 min, and extension at 72 °C for 2 min for 35 cycles. In parallel a C-terminal truncation mutant (ΔCIC; primer 15 and 16) was generated that lacked the cysteines at position 507 and 509. The resulting cDNAs were inserted in the pEGFP-C1 vector (Clontech). Using the QuickChange site-directed mutagenesis approach (Stratagene) amino acid substitution mutants K60A (primers 1 and 2), D64A (primers 3 and 4), E98A (primers 5 and 6), K112A (primers 7 and 8), Y115A (primers 9 and 10), C379A (primers 11 and 12), and C455A (primers 13 and 14) were generated. See Table 1 for primer sequences.

In vitro aminoacylation assay

Mock and SIN-1-treated (8 h) Jurkat cells were harvested by centrifugation (1,000xg for 5 minutes at room temperature), resuspended in 100 mM KCl, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, 2.5 U/µl RNasin (Promega), and 1 mM DTT and lysed by five 30-s pulses in an ice-cold sonication waterbath (Bioruptor). The resulting homogenates were cleared by centrifugation at 21,000xg for 30 min at 4 °C. To study tRNA aminoacylation lysates (10x10^6 Jurkat cell equivalents) were incubated for 1.5 hours at 37 °C in 50 µl reactions containing 100 mM KCl, 1 mM $MgCl_2$,

1 mM DTT, 1 mM ATP, 20 mM Tris-HCl, pH 7.4, [^{14}C] histidine (Amersham), and 2.5 U/µl RNasin. TRIzol was used to isolate total RNA from the aminoacylation reaction mixtures and the RNA was separated in 6.5% polyacrylamide 0.1 M NaOAc, pH 5.0, 7.5 M urea gels [15]. Subsequently, RNA was transferred to Hybond-N membranes (Amersham) and [^{14}C]-labeled RNA was visualized by phosphorimaging. Anti-sense [^{32}P]labeled-tRNA^{His} and [^{32}P]labeled-5.8S rRNA probes were synthesized as described previously and used in northern blot hybridization [16]. The membranes were blocked for 1 hour at 65 °C with hybridization mix containing 1x Denhardt's solution, 0.1 mg/ml herring sperm DNA, 0.2% SDS, and 6x SSC. Subsequently, membranes were incubated overnight with [^{32}P]labeled anti-sense tRNA^{His} or 5.8S rRNA at 65 °C. The membranes were washed twice with 2x SSC and 0.1% SDS before hybridized probes were visualized by phosphorimaging. Signals were quantified by Quantity One software v4.6.9 (Bio-Rad).

Detection of autoantibodies to crosslinked HisRS

Patient sera with antibodies reactive with the bacterially expressed recombinant human HisRS [14] protein were incubated with 0.1, 1, 10, or 100 ng purified recombinant HisRS per 100 µl serum diluted 400 to 50,000-fold in PBS. After 30 min incubation at room temperature, sera were centrifuged at 16,000xg for 30 min at 4 °C. The anti-Jo-1 depleted and control anti-Jo-1 sera were used for immunoblotting as described above.

Results

HisRS crosslinking upon SIN-1 treatment of Jurkat cells

To investigate whether HisRS is post-translationally modified under ROS/RNS conditions, Jurkat cells were cultured in the presence of SIN-1 to generate $ONOO^-$. SIN-1 is a metabolite of molsidomine and decomposes spontaneously in solution in the presence of O_2, releasing $NO^\bullet$ and $O_2^{\bullet-}$ that can react and form $ONOO^-$.

Cultured Jurkat cells were subjected to 0.4 mM SIN-1 for several time periods. Subsequently, Jurkat cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. The formation of a high molecular weight HisRS isoform (HisRS*) was observed in SIN-1-treated Jurkat cells (Fig. 1A). This polypeptide was detected both with an anti-Jo-1 positive patient serum and with a single chain antibody (scFv) directed to HisRS [17]. Since HisRS is known to form homodimers *in vivo* and the molecular mass (100 – 110 kDa) of HisRS* appeared to correspond to that of a HisRS homodimer, the high molecular weight species might correspond to a crosslinked homodimer. The formation of HisRS* appeared to be relatively efficient leading to an approximately 1:1 ratio between HisRS and HisRS* after 8 hours. Because the sample preparation was performed under reducing and denaturing conditions, the putative linkage between the HisRS polypeptides cannot be due to disulfide bond formation (Fig. 1A).

Cell death- and cell type-independent HisRS crosslinking upon SIN-1 treatment

It has been shown that peroxynitrite can elicit either apoptosis or necrosis, depending on its intracellular concentration [18]. To investigate whether HisRS crosslinking is associated with the induction of cell death, we determined the induction of apoptosis or necrosis by SIN-1. Neither morphological changes that are characteristic for cell death nor prototypical molecular alterations for apoptosis (U1-70k cleavage) or necrosis (topoisomerase I cleavage) [19] were observed in Jurkat cells treated with SIN-1 (Supplementary Fig. 1). Even with the highest concentrations of SIN-1 that were used (1.6 mM; see Fig. 1B) no detectable induction of cell death was observed in cells exposed for 8 hours. Note that the highest concentrations of SIN-1 (0.8 mM and 1.6 mM) led to a slight retardation of crosslinked HisRS in SDS-PAGE (indicated by HisRS**). Similar experiments with other cell lines (HEp-2 and HeLa, two epithelial cell lines, Molt-4, a lymphoblast line, and TE671, rhabdomyosarcoma cells) demonstrated that the observed crosslinking of HisRS upon SIN-1 treatment is not a unique feature of Jurkat cells, because the same phenomenon was observed in the other cell lines (data not shown). To investigate whether other Class II aminoacyl-tRNA synthetases and other autoantigenic proteins showed similar changes after SIN-1 treatment, lysates from Jurkat cells treated with 0.4 mM SIN-1 for 8 hours were analyzed by immunoblotting using antibodies specific for alanyl-tRNA synthetase (PL12), threonyl-tRNA synthetase (PL7), phenylalanyl-tRNA synthetase (Zo), U1A, a U1 snRNP associated protein, and Rrp40, a core exosome protein. For none of these proteins changes in the electrophoretic mobility in SDS-PAGE comparable to that of HisRS were observed (Fig. 1C and data not shown).

ROS/RNS scavengers inhibit HisRS crosslinking

As described above SIN-1 decomposition forms $O_2^{\bullet-}$ and $NO^\bullet$ and leads to the generation of $ONOO^-$. We hypothesized that at least one of these compounds contributed directly to the generation of

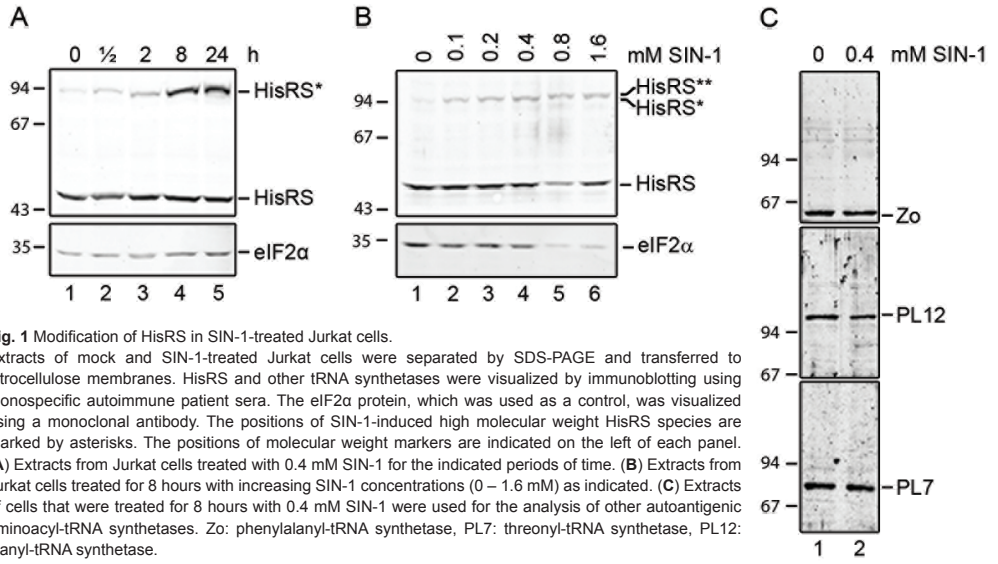


Fig. 1 Modification of HisRS in SIN-1-treated Jurkat cells.
Extracts of mock and SIN-1-treated Jurkat cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. HisRS and other tRNA synthetases were visualized by immunoblotting using monospecific autoimmune patient sera. The eIF2α protein, which was used as a control, was visualized using a monoclonal antibody. The positions of SIN-1-induced high molecular weight HisRS species are marked by asterisks. The positions of molecular weight markers are indicated on the left of each panel. (**A**) Extracts from Jurkat cells treated with 0.4 mM SIN-1 for the indicated periods of time. (**B**) Extracts from Jurkat cells treated for 8 hours with increasing SIN-1 concentrations (0 – 1.6 mM) as indicated. (**C**) Extracts of cells that were treated for 8 hours with 0.4 mM SIN-1 were used for the analysis of other autoantigenic aminoacyl-tRNA synthetases. Zo: phenylalanyl-tRNA synthetase, PL7: threonyl-tRNA synthetase, PL12: alanyl-tRNA synthetase.

the crosslinked HisRS dimer. To shed more light on this, scavengers for superoxide and peroxynitrite were added to the Jurkat cultures during SIN-1 treatment. The cells were treated with SIN-1 in the presence of Tiron (a superoxide scavenger) [20] or uric acid (a peroxynitrite scavenger) [21] (Fig. 2). Jurkat cells treated with either 1000 µM Tiron or 100 µM uric acid alone showed no effects on cell morphology or cell survival (Supplementary Fig. 2). The exposure of the cells to these compounds also did not lead to detectable changes of HisRS, whereas SIN-1 alone as before stimulated the crosslinking of HisRS. The crosslinking of HisRS appeared to be repressed in a concentration dependent manner by both scavengers, although the repression by Tiron was more pronounced than that in the presence of uric acid (Fig. 2). These results are consistent with the involvement of superoxides in the generation of the HisRS crosslinks.

Covalent crosslinking of homodimeric HisRS

The aminoacyl-tRNA synthetases have been grouped into two different classes depending on their conformation, conserved domains, and activity [22]. Crystal structure analysis and native gel electrophoresis confirmed that the HisRS protein is a Class II aminoacyl-tRNA synthetase and is able to form dimers [23,24]. To investigate whether the SIN-1-induced HisRS species indeed represents a crosslinked homodimer, an EGFP-HisRS fusion protein was expressed in Jurkat cells. The expression of the fusion protein was confirmed by western blotting using both anti-EGFP and anti-Jo-1 antibodies (Fig. 3). Like the endogenous HisRS protein also the EGFP-tagged HisRS appeared to be crosslinked upon SIN-1 treatment. Interestingly, when the material from SIN-1 treated cells was probed with the anti-Jo-1 antibody an additional crosslinking species was observed, which may represent a crosslinked dimer composed of non-tagged HisRS and EGFP-HisRS. These data strongly support the hypothesis that the crosslinked HisRS species formed during SIN-1 treatment represent crosslinked HisRS dimers.

Identification of the amino acids involved in HisRS crosslinking

The formation of peroxynitrite upon SIN-1 treatment elicits both oxidative and nitrosative stress resulting in the crosslinking of HisRS dimers. To obtain more insight into the amino acids that are involved in crosslinking, several residues selected based upon structural data (personal communication Prof. Dr. P. Schimmel; [25]) were replaced by alanine. In addition, a C-terminal truncation mutant was generated. The EGFP-tagged HisRS mutants K60A, D64A, E98A, K112A, Y115A, C379A, and C455A were all able to form crosslinked dimers after SIN-1 treatment, although the efficiency of dimerization seems to be affected by some of these mutations, which is substantiated by the differences in the ratio between crosslinked EGFP-HisRS homodimer and crosslinked EGFP-HisRS – endogenous HisRS heterodimer. In contrast, crosslinking of the HisRS fusion protein was totally abolished by the deletion of the three most C-terminal amino acids (ΔCIC) (Fig. 4A). The exact position of the mutated residues and their side chains are depicted in the structural model for the human HisRS protein (lacking the N-terminal

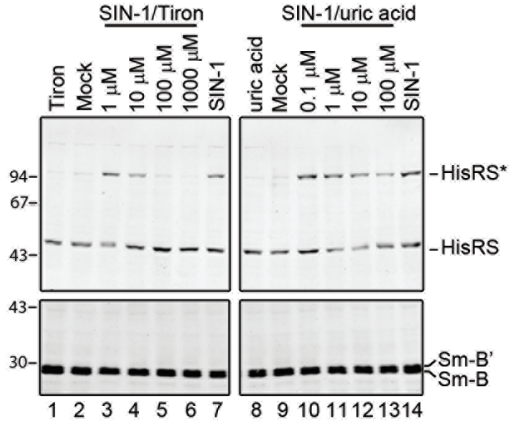


Fig. 2 Effects of ROS/RNS scavengers on SIN-1-induced HisRS crosslinking.
Jurkat cells were treated for 8 hours with 0.4 mM SIN-1 in the presence of different concentrations of Tiron (lanes 3-6) or uric acid (lanes 10-13) as indicated, or only with either 1000 µM Tiron (lane 1), 100 µM uric acid (lane 8), 0.4 mM SIN-1 (lanes 7, 14) or buffer (lanes 2, 9). Extracts of treated cells were analyzed by immunoblotting using a monospecific anti-Jo-1 patient serum or a patient serum reactive with the Sm-B/B' proteins, which was used as a control. The positions of molecular weight markers are indicated on the left.

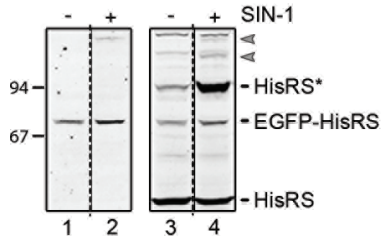


Fig. 3 Crosslinking of EGFP-HisRS in SIN-1-treated Jurkat cells.
Jurkat cells were transfected with cDNA constructs encoding EGFP-HisRS by electroporation. Fourty-eight hours post-transfection the cells were exposed to 0.4 mM SIN-1 (+) or buffer (–) for 8 hours and extracts were analyzed by immunoblotting. The blots were stained with either mouse monoclonal anti-EGFP antibodies (lanes 1, 2) or anti-Jo-1 antibodies (lanes 3, 4). High molecular weight signals (approximately 135 kDa and 160 kDa) in material from SIN-1 treated cells are indicated with arrowheads.

60 amino acids), which was generated by the YASARA program (Fig. 4B,C). These results indicate that the C-terminal residues -C-I-C are essential for the formation of the crosslinked HisRS dimer.

Effects of SIN-1 treatment on cell growth and translation

To study the effects of SIN-1-induced crosslinking of HisRS on its function, cell growth rates, the efficiency of translation, and the aminoacylation of $tRNA^{His}$ were determined. Jurkat cells were cultured in the presence of 0.4 mM SIN-1 and at different time points (up to 72 hours) the number of cells was determined. The results showed that cell growth was indeed impaired by the exposure to SIN-1 (Fig. 5A). The reduced cell growth rates might be due to the inhibition of translation as a result of the impairment of the function of HisRS and/or other factors involved in protein synthesis. To investigate the effects on the efficiency of translation, the incorporation of [^{35}S]methionine in proteins in (SIN-1 treated) Jurkat cells was determined. The results showed that protein synthesis during a period of 2 or 4 hours after culturing the cells for 6 hours in the presence of SIN-1 was reduced by approximately 40% (Fig. 5B). The next question we tried to answer was whether crosslinking of HisRS dimers would result in decreased aminoacylation of $tRNA^{His}$. Lysates from mock and SIN-1-treated Jurkat cells were used in *in vitro* aminoacylation reactions. Reaction products were analyzed by denaturing gel electrophoresis and autoradiography or northern blot hybridization. The total amounts of $tRNA^{His}$ and 5.8S rRNA, which was analyzed as a control, did not significantly differ between mock and SIN-1 treated Jurkat cells. More importantly, the HisRS activity was not or hardly altered as a result of the SIN-1-induced crosslinking (Fig. 5C). As an additional control the aminoacylation reaction with lysate from mock-treated cells was incubated with a 1,000-fold excess of unlabelled histidine (Ctrl), which, as expected, completely abolished the aminoacylation of $tRNA^{His}$. These results suggest that the aminoacylation of $tRNA^{His}$ remains unaffected and thus cannot explain the impaired growth and inhibition of translation detected after SIN-1 treatment.

Autoantibodies specifically targeting the crosslinked HisRS dimer are not detectable

Dimerization, oligomerization and crosslinking of proteins may contribute to the formation of pathogenic structures [26-28]. Such structures may generate neo-epitopes and initiate an autoimmune response. To address the question whether autoantibodies specifically directed against the crosslinked HisRS dimer are present in myositis sera, a longitudinal series of serum samples from an anti-Jo-1-positive patient, collected during a period of 13 years, was used. First, the reactivity of these samples with HisRS from both mock and SIN-1 treated Jurkat cells was assessed by immunoblotting. The ratio between the signals for HisRS and HisRS* did not detectably change during the course of the disease (Supplementary Fig. 3A). To rule out the possibility that crosslinked dimer specific antibody signals are masked by other anti-Jo-1 antibody signals, the autoantibody reactivities were determined using a competition assay as described in Materials and methods. The purified, bacterially expressed recombinant human HisRS protein efficiently competed for the binding

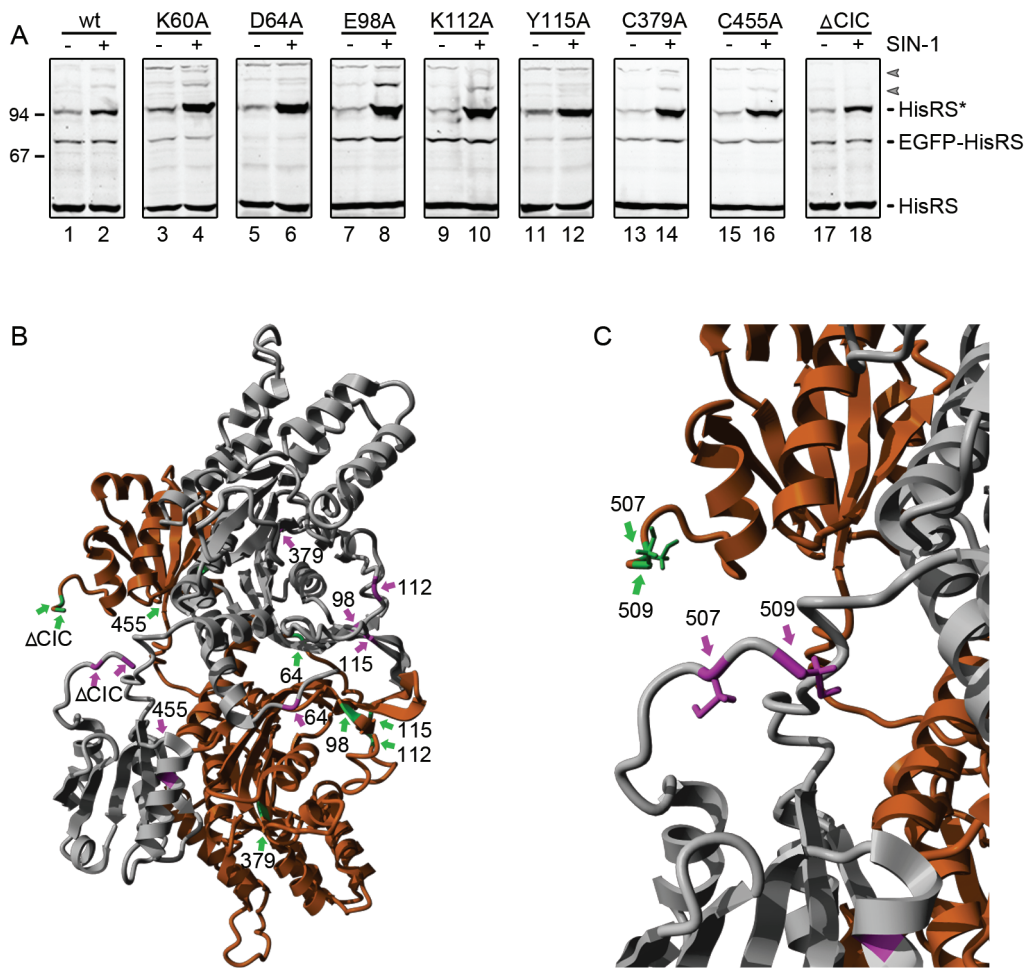


Fig. 4 SIN-1-induced crosslinking of HisRS mutants.
(**A**) Jurkat cells were transfected with cDNA constructs encoding the EGFP-tagged wild-type (wt), K60A, D64A, E98A, K112A, Y115A, C379A, C455A single amino acid substitution mutants, or the C-terminally truncated (ΔCIC) EGFP-HisRS protein and 48 hours post-transfection the cells were mock (−) or SIN-1-treated (+) for 8 hours. Cell extracts were analyzed by immunoblotting using a monospecific anti-Jo-1 patient serum. The HisRS fusion proteins are indicated with EGFP-HisRS, whereas endogenous HisRS and crosslinked HisRS are labelled with HisRS and HisRS*, respectively. The crosslinked EGFP-HisRS species are indicated with arrowheads. (**B**) Structural model of the homodimeric HisRS protein (lacking the N-terminal 60 amino acids), with subunits A and B in grey and brown, respectively. The positions of the mutated amino acids are indicated in magenta (one subunit) and green (other subunit). Note that K60 is not included in this model. (**C**) Enlargement of the C-terminal region of both subunits with the truncated cysteines and their side chains in magenta and green.

of anti-Jo-1 antibodies to HisRS from Jurkat cells, and the competition for the binding to HisRS* was efficient as for the binding to HisRS (Supplementary Fig. 3B). Using this approach, thirty-five anti-Jo-1 sera of established myositis patients were analyzed. However, in none of the tested sera specific reactivity to the crosslinked HisRS dimer was detected (results not shown). Taken together, these results do not provide evidence for the existence of specific autoantibodies to the crosslinked HisRS dimer in established myositis patients.

Discussion

Aminoacyl-tRNA synthetases fulfill an important role in protein synthesis by conjugating the tRNAs to their cognate amino acids via an esterification reaction. As a consequence, translation can be impaired severely if tRNAs are mischarged or their aminoacylation activity is dysregulated in some way. Here, we show that elevated levels of reactive oxygen/nitrogen species lead to a covalent linkage between HisRS molecules. The homodimers are resistant to reducing agents and their association depends on the three most C-terminal residues (CIC) of HisRS. The tRNA

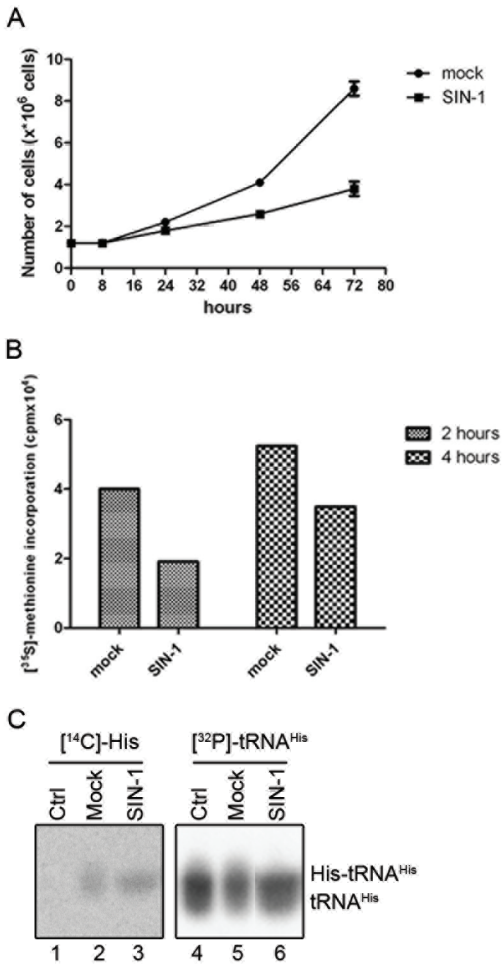
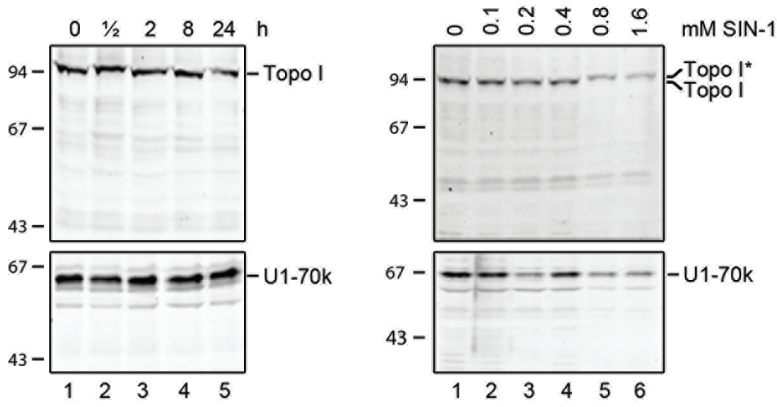


Fig. 5 The effects of SIN-1 treatment on cell growth, translation, and $tRNA^{His}$ aminoacylation.
(**A**) Logarithmically growing Jurkat cells were exposed to SIN-1 (0.4 mM) or buffer (mock) for 0 – 72 hours and at several time points the number of cells was determined. The graphs show the mean and standard deviation of experiments performed in triplicate. (**B**) Cells were mock or SIN-1 treated for 6 hours prior to the addition of [^{35}S]-methionine. Cells were harvested after 2 hours and 4 hours and cell lysates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The total [^{35}S]-methionine signals were quantified using phosphorimaging. Finally, β-actin was visualized by incubating the blots with a monoclonal anti- β-actin antibody. The [^{35}S]-methionine signals were normalized based upon total β-actin signals. (**C**) Cell lysates from mock and SIN-1 (0.4 mM) treated Jurkat cells were used in an *in vitro* aminoacylation assay containing ^{14}C-histidine. As a control (Ctrl) extract of mock-treated cells was supplemented with a 1,000-fold excess of unlabelled histidine. The ^{14}C signals were visualized by phosphorimaging. Total $tRNA^{His}$ was visualized by northern blot hybridization using a [^{32}P]-labeled antisense $tRNA^{His}$ probe and phosphorimaging.

aminoacylation activity of HisRS did not seem to be affected by this crosslink.

Cells have to cope with all kinds of endo- and exogenous stress factors, and this may lead to cell death when defense mechanisms are inadequate or failing. Under such conditions, elevated levels of ROS/RNS may be generated. Also during an inflammation a respiratory burst will lead to enhanced oxidative/nitrosative stress. The formation of ROS/RNS in cells or tissues has been implicated in a number of (patho) physiological processes. Low levels of radicals have been suggested to act as messenger molecules and to regulate cellular processes, whereas excessive amounts can overload the antioxidant mechanisms and result in the modification of macromolecules. Inflammatory cells that infiltrate endomysial tissue in PM and DM patients were shown to produce increased nitric oxide synthase (NOS) levels [12], which may lead to higher NO concentrations. We recently observed that HisRS is oxidatively modified in Jurkat cells upon H_2O_2-induced stress. In addition we showed that H_2O_2-induced stress leads to the formation of disulfide bonds linking the C-termini of both subunits of the HisRS dimer (Van Dooren et al., manuscript submitted for publication). In the present study we have shown that the same cysteines at the C-termini are involved in subunit crosslinking as a result of the exposure to another ROS/RNS generator, SIN-1. In contrast to the H_2O_2-induced crosslinks, the SIN-1-induced crosslinks appear to be resistant to reducing agents. The inhibition of SIN-1-induced crosslinking by both a superoxide and a peroxynitrite scavenger suggests that peroxynitrite, which can be formed by the reaction of NO with $O_2^{\bullet-}$, plays an important role. Peroxynitrite is in equilibrium with peroxynitrous acid (ONOOH) and either one can undergo direct reactions with biomolecules, including thiols associated with these biomolecules. Alternatively, in biological systems peroxynitrite can rapidly react with carbon dioxide, which is in equilibrium with the bicarbonate anion, leading to the formation of nitrogen dioxide and carbonate radicals. Currently, it is unknown whether these radicals or direct peroxynitrite reaction products can result in the formation of covalent bonds between the C-terminal cysteines of HisRS. Besides linkages formed between the side chains of two cysteines, covalent bonds between one cysteine and another amino acid in the other subunit might be possible as well. Indeed, cysteine-tyrosine thioether crosslinks and cysteine-dehydroalanine (dehydroalanine may result from the oxidation of serine) crosslinks have been reported [29-32]. However, the involvement of the latter linkages in the HisRS crosslinks is very unlikely, because the deletion of the C-terminal CIC amino acids not only abrogated the EGFP-HisRSΔCIC – EGFP-HisRSΔCIC crosslink, but also the EGFP-HisRSΔCIC – endogenous HisRS crosslink. If such linkages would be involved in



Supplementary Fig. 1 Detection of cell death markers in SIN-1 treated Jurkat cell lysates.
Protein extracts of mock and SIN-1 treated Jurkat cells were fractionated by SDS-PAGE and analyzed via immunoblotting. Topoisomerase I (Topo I) and U1-70k were visualized by immunoblotting using patient sera containing antibodies against these proteins. (**A**) Cells were treated with 0.4 mM SIN-1 for the indicated periods of time. (**B**) Jurkat cells were treated for 8 hours with different SIN-1 concentrations as indicated.

dimeric Jo-1 crosslinking, the formation of the latter would be expected to be unaffected by the deletion of the C-terminal CIC element in the EGFP-tagged protein. Further studies will be required to unravel the chemical nature of the SIN-1-induced bond between the HisRS subunits. It should be noted that our results do not completely rule out the possibility that one or more non-HisRS protein(s) are crosslinked with the EGFP-HisRS protein. To unambiguously proof that the SIN-1-induced crosslinked HisRS species represent HisRS homodimers, the molecular composition of these molecules has to be elucidated, e.g. by mass spectrometry.

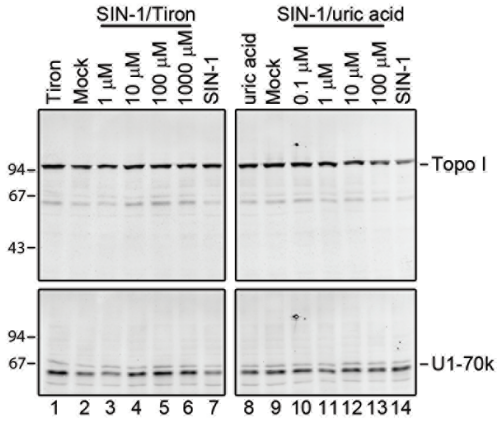
None of the other tested (autoantigenic) proteins showed a similar effect upon treatment of the cells with SIN-1, which suggests that the crosslink is specific for HisRS, which is consistent with the presence of the two cysteines in the flexible C-terminal tail of HisRS.

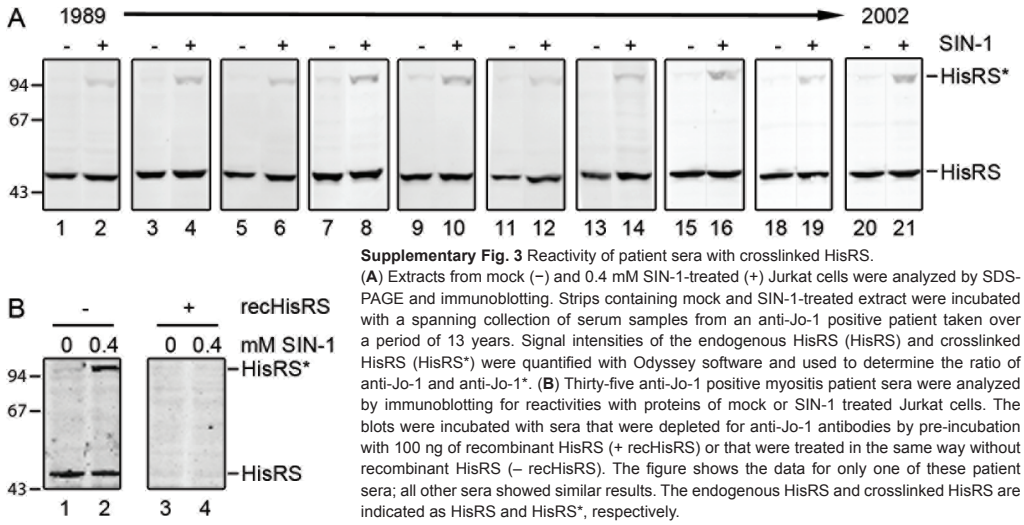
In contrast to the previously reported SIN-1-induced cell death [18], the Jurkat cells used in our studies did not display the typical features of apoptosis or necrosis, like U1-70k and topoisomerase I cleavage [19]. Nevertheless, the cells appeared to be stressed, since both cell growth and protein translation were affected by the SIN-1 treatment. The tRNA aminoacylation activity of HisRS from treated cells was comparable to that of control cells, which suggests that the retarded cell growth and decreased rate of protein synthesis were not due to disturbed $tRNA^{His}$ aminoacylation.

Oxidatively modified proteins have been proposed to initiate an immune response when they are exposed to the immune system. Since ROS/RNS often are associated with necrotic loci and infiltrating inflammatory cells are commonly found in skeletal muscle tissue of myositis patients this might be a feasible mechanism for the generation of myositis-associated autoimmunity. Indeed, the ROS/RNS-induced HisRS modification described here may result in the generation of a neo-epitope and thus facilitate the generation of an autoimmune response. To study whether autoantibodies specifically recognizing neo-epitopes in the crosslinked HisRS dimer are present in patient sera, it is necessary to analyze predisease sera, since such autoantibodies are expected to be produced early in the disease. However, we were unable to obtain such sera, because most individuals are visiting the clinic only when clinical symptoms become manifest.

In conclusion, our data reveal a novel peroxynitrite or peroxynitrite-derived radical-induced crosslink in homodimeric HisRS that depends on the C-terminal cysteines and raise new questions on the chemical nature and physiological role of this crosslink. Although HisRS crosslinking does not appear to affect the $tRNA^{His}$ aminoacylation, it may play a role in the preservation of enzyme activity during stress by preventing the dissociation of the HisRS dimer.

Supplementary Fig. 2 Detection of cell death markers in ROS/RNS scavenger treated Jurkat cells.
Jurkat cells were treated for 8 hours with 0.4 mM SIN-1 in the presence of different concentrations of either Tiron or uric acid as indicated. As a control cells were treated with 0.4 mM SIN-1 (lanes 7, 14), 1000 µM Tiron (lane 1) or 100 µM uric acid (lane 8) alone. Topoisomerase I (Topo I) and U1-70k were visualized by immunoblotting using patient sera.





Supplementary Fig. 3 Reactivity of patient sera with crosslinked HisRS.
(**A**) Extracts from mock (−) and 0.4 mM SIN-1-treated (+) Jurkat cells were analyzed by SDS-PAGE and immunoblotting. Strips containing mock and SIN-1-treated extract were incubated with a spanning collection of serum samples from an anti-Jo-1 positive patient taken over a period of 13 years. Signal intensities of the endogenous HisRS (HisRS) and crosslinked HisRS (HisRS*) were quantified with Odyssey software and used to determine the ratio of anti-Jo-1 and anti-Jo-1*. (**B**) Thirty-five anti-Jo-1 positive myositis patient sera were analyzed by immunoblotting for reactivities with proteins of mock or SIN-1 treated Jurkat cells. The blots were incubated with sera that were depleted for anti-Jo-1 antibodies by pre-incubation with 100 ng of recombinant HisRS (+ recHisRS) or that were treated in the same way without recombinant HisRS (– recHisRS). The figure shows the data for only one of these patient sera; all other sera showed similar results. The endogenous HisRS and crosslinked HisRS are indicated as HisRS and HisRS*, respectively.

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Chapter 7

A biomarker for Inclusion Body Myositis: anti-Mup44, the first IBM-associated autoantibody

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Abstract

Objective

Inclusion body myositis (IBM) is the most common acquired muscle disease in people over 45. Both degenerative and autoimmune features are involved in its pathogenesis. Specific antigens to which the autoimmune response in IBM is directed have not been described so far. To better understand IBM pathophysiology and to facilitate the diagnosis of this disease human muscle tissue was used to identify IBM-specific or -associated autoantigens.

Methods

Western blots were prepared with extracts from human hamstring muscle or from cultured cell lines. The reactivity of IBM patient and control sera with this material was assessed by immunoblotting.

Results

A mature skeletal muscle antigen of 44 kDa, Mup44, was identified as an autoantibody target in IBM. Mup44 was recognized by 23% of IBM patient sera. Only a limited number of sera from other myositis subclasses, polymyositis and dermatomyositis, was reactive with Mup44 (2% and 0% reactivity, respectively). In addition, sera from most other autoimmune diseases tested and sera from healthy individuals showed no reactivity with Mup44, although anti-Mup44 reactivity was detected in some systemic lupus erythematosus (SLE) and Sjögren's syndrome (SjS) sera (9% and 7%, respectively).

Interpretation

For the first time, an IBM-associated autoantigen, designated Mup44, was identified. Mup44 is expressed in human skeletal muscle, but not in Jurkat, human T lymphocyte cells nor in C2C12, mouse myoblast cells. Anti-Mup44 antibodies are found with a relatively high frequency in IBM sera and in some sera of SLE and SjS patients. Mup44 may facilitate the diagnosis of IBM.

Introduction

Sporadic Inclusion Body Myositis (IBM) is the most common muscle disease in people over 45. It is clinically characterized by insidious onset of muscle weakness, without evidence of extramuscular manifestations, finally causing serious disability [1-3]. IBM belongs to the idiopathic inflammatory myopathies (IIM), collectively called myositis, which are defined by an acquired muscle weakness and the presence of inflammatory infiltrates in skeletal muscle tissue in the absence of a clear cause. Myositis is classified into dermatomyositis (DM), polymyositis (PM) and IBM [4-8].

PM and DM are considered to be autoimmune diseases. The presence of inflammatory cell infiltrates in the affected muscles, the frequent occurrence of autoantibodies and the association with other autoimmune diseases suggest that their generation is immune mediated with an important role for both $CD4^+$ and $CD8^+$ T lymphocytes [7-9]. High-titer autoantibodies are detected in the sera of 60-80% of PM and DM patients. They are classified in myositis-specific autoantibodies (MSA) such as anti-Jo-1, anti-Mi-2 and anti-SRP antibodies, which are highly specific for myositis and not or only sporadically occurring in other rheumatic diseases, and in myositis-associated antibodies (MAA), which are also frequently found in several other autoimmune diseases [7,10-13]. The most common MSA is anti-Jo-1, which is found in 20-30% of PM and DM patients and its production may precede the symptoms of myositis (reviewed in [7]). The presence of specific autoantibodies in PM and DM is correlated with specific clinical phenotypes, e.g. anti-Jo-1 is a marker for the anti-synthetase syndrome, consisting of PM or DM in combination with extramuscular manifestations like Raynaud's phenomenon, interstitial lung disease and polyarthritis [7].

IBM on the other hand is hypothesized to be a primarily degenerative disease with secondary immune components [1,14]. Cytoplasmic accumulation of several aggregate-prone proteins (e.g. β-amyloid, αB-crystallin, TAR DNA binding protein-43) including some which are typically associated with neurodegenerative diseases such as Alzheimer's disease, has been reported as a characteristic feature of IBM muscle [14-18]. Endoplasmic reticulum (ER) stress, proteasome inhibition, mitochondrial abnormalities and oxidative stress are seen as major determinants for the development of the disease [1,14,19,20]. At the same time IBM is a muscle-specific autoimmune disease in which clonal expansion of T- and B-cells plays a part [1,2,9,20,21]. Although MAA such as anti-Ro52 and anti-La have been reported in IBM, no IBM-specific autoantibodies have been identified so far. In addition, known MSAs such anti-Jo-1 and anti-Mi-2 are only occasionally found in IBM [10,11,22,23]. It was proposed that autoantibodies are less frequently found in IBM than in PM and DM and that the presence of a MSA (anti-Jo-1 autoantibody) may even exclude the diagnosis of IBM [7,11,24].

Based upon the hypothesis that the autoimmune response in IBM is directed towards muscle-specific antigens, we investigated in this study the reactivity of IBM patient sera with human skeletal muscle tissue extracts. A muscle-specific polypeptide of 44 kDa appeared to be recognized by 23% of IBM sera and autoantibodies to this protein display a rather high level of disease-specificity.

Methods

Serum samples

Serum samples from a group of well-characterized inflammatory myopathy patients described by Abdo *et al.* [25] (31 IBM, 48 PM and 24 DM patients; diagnosed according to established international criteria [25,26]) were used in this study. Serum samples were stored at -80 °C for long term storage; at the time of analysis NaN_3 was added (final concentration 0.02%) to 50 µl serum aliquots, which were stored at 4 °C. Separate groups of serum samples from IBM patients from the study of Hengstman *et al.* [11] and from Dr. Badrising (Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands) were also studied.

To assess the disease specificity of the detected antibodies, sera from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), scleroderma (Scl), PM-scleroderma overlap (PM-Scl) and primary Sjögren's syndrome (SjS) [27,28] as well as serum from healthy individuals obtained from the Sanquin Blood Supply Foundation (Nijmegen, The Netherlands) were analyzed.

Muscle extracts

Human healthy hamstring muscle obtained from reconstructive surgery (a kind gift of Dr. Snoeck, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands) was frozen in liquid nitrogen and stored at -80 °C. Small pieces of frozen muscle tissue were pulverized with a Microdismembrator (Braun Biotech International, Melsungen, Germany), resuspended in 10 volumes of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 10 mM DTT, 0.5 mM PMSF and Protease

Table 1. Prevalence of anti-Mup44 autoantibodies in myositis patient sera

	# of sera tested	# of sera recognizing Mup44	% of sera recognizing Mup44
IBM[a]	31	9	29
DM[a]	24	0	0
PM[a]	47	1	2
IBM[b]	28	4	14
IBM[c]	32	8	25

[a]Myositis serum cohort 1 [25]; [b]IBM serum cohort 2 [11]; [c]IBM serum cohort 3 [Dr. Badrising, Leiden University Medical Center, Leiden, The Netherlands]

Inhibitor Cocktail (Roche, Mannheim, Germany)) at 4 °C and centrifuged for 15 minutes at 21,000 *g*. The supernatant was used for SDS-PAGE followed by western blot analysis. Protein concentration of the muscle extracts was determined by the BCA assay (Pierce, Rockford, USA).

Jurkat and C2C12 cell lysates

Human Jurkat T-lymphocyte and mouse C2C12 myoblast cells were cultured according to standard procedures. C2C12 cells were grown to confluency and cultured for 5 days with DMEM containing 2% horse serum for differentiation into myotubes. Jurkat cells were pelleted by centrifugation, washed with PBS, lysed by sonication in lysis buffer (50 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 0.05% NP-40, 0.5 mM PMSF and Protease Inhibitor Cocktail) and centrifuged at 21,000 *g*. C2C12 myotubes were washed with PBS, scraped in lysis buffer, followed by sonication and centrifugation. The resulting supernatants were used for SDS-PAGE followed by western blot analysis. Protein concentration of the lysates was determined by the BCA assay.

Western blot analysis

200-300 µg of human muscle extract or 100-200 µg of Jurkat or C2C12 cell lysate was separated by electrophoresis in mini-Protean 12% SDS-PAGE gels (Bio-Rad, Hertfordshire, United Kingdom), using the full width of the gels, and blotted to nitrocellulose membranes. Membranes were cut into strips of 3-4 mm wide, blocked with 5% milk powder in PBS-0.1% Tween-20 (PBS-T) and incubated with human patient serum, diluted 1:1000 in 5% milk-PBS-T. Bound antibodies were visualized using goat-anti-human-IRDye800 labeled secondary antibody (Rockland, Gilbertsville, USA) and scanned with the Odyssey system (LI-COR Biosciences, Lincoln, USA).

Table 2. Prevalence of anti-Mup44 autoantibodies in autoimmune patient sera

	# of sera tested	# of sera recognizing Mup44	% of sera recognizing Mup44
IBM[a]	91	21	23
DM[a]	24	0	0
PM	47	1	2
PM-Scl	22	0	0
Scl	16	0	0
RA	22	0	0
SLE	43	4	9
SjS	28	2	7
NHS[b]	32	0	0

[a]Total of all tested IBM sera; [b]Sera from healthy individuals.

Serological data

Ro52, Ro60, La and Jo-1 autoantibody reactivity has been analyzed by ELISA as described [10,11].

Results

Identification of Mup44 in skeletal muscle extracts

To investigate the presence of (IBM-specific) autoantibody targets in human skeletal muscle tissue, human hamstring extracts were separated by SDS-PAGE, blotted to nitrocellulose and incubated with serum from IBM patients. As shown in Figure 1A several muscle polypeptides were recognized by the antibodies in these patient sera. Although different sera were reactive with different muscle antigens, one prominent band appeared to be commonly detected

Table 3. Co-occurrence of anti-Mup44 autoantibodies with anti-Ro52, anti-Ro60, anti-La and anti-Jo-1 autoantibodies in IBM patient sera

	IBM sera[a]	Anti-Mup44 positive	Anti-Mup44 negative
# of sera	58	11	47
Anti-Ro52	10 (17%)	2 (18%)	8 (17%)
Anti-Ro60	9 (16%)	1 (9%)	8 (17%)
Anti-La	6 (10%)	1 (9%)	5 (11%)
Anti-Jo-1	1 (2%)	0 (0%)	1 (2%)

[a]IBM sera from cohort 1 [11] and cohort 3 (Dr. Badrising, Leiden University Medical Center, Leiden, The Netherlands) for which Ro52, Ro60, La and Jo-1 autoantibody data were obtained.

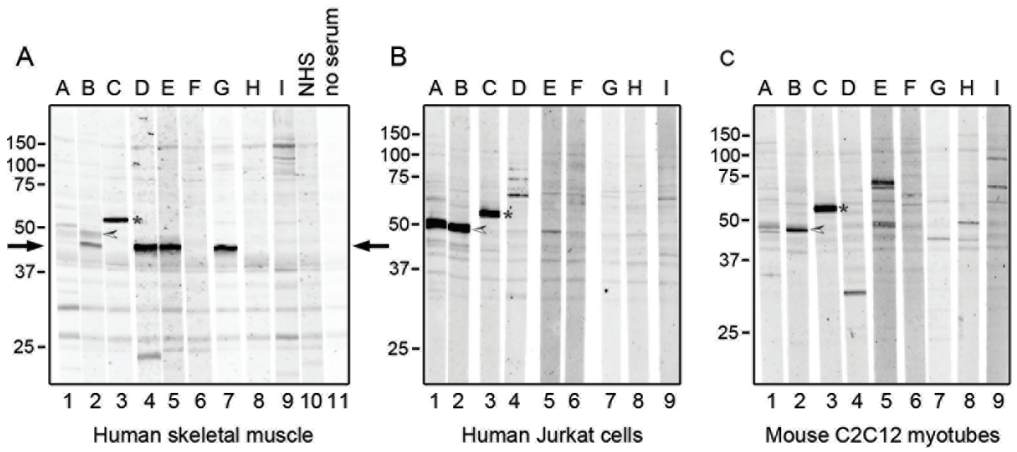

Fig. 1 Human skeletal muscle contains a novel IBM autoantigen.
(A) A human skeletal muscle tissue extract was separated by SDS-PAGE and blotted to nitrocellulose membranes. The blot strips were incubated with IBM patient sera (A-I) and a healthy control serum (NHS), followed by detection of the bound antibodies by incubation with an IRDye800-labeled anti-human secondary antibody. Bound antibodies were visualized with the Odyssey infrared imaging system. Arrows indicate the Mup44 muscle antigen detected by IBM sera. (**B-C)** Human Jurkat cell lysate (B) and mouse C2C12 myotube cell lysate (C) were analyzed in the same way as in panel A. Asterisks and arrowheads in panels A, B and C indicate putatively identical antigens. The positions of molecular weight markers are indicated on the left of each panel.

by several IBM sera (Fig. 1A, arrow). Four out of 9 IBM sera were reactive with this polypeptide with a molecular weight of 44 kDa.

Interestingly, while some antigens detected in muscle extracts by IBM sera were also identified using lysates from cultured human cell lines such as Jurkat cells (Fig. 1B), the prominent 44 kDa polypeptide appeared to be detectable only in the material from skeletal muscles. Western blotting with differentiated mouse C2C12 myotube cell lysates and mouse skeletal muscle extracts indicated that the antigen recognized by the IBM sera is specifically present in skeletal muscle samples, since it could not be detected in the myotube cell culture lysates (Fig. 1C), whereas it was detected in mouse skeletal muscle extracts (data not shown). Immunoblotting using adjacent blot strips confirmed that the 44 kDa antigen detected by different IBM patient sera (Fig. 2) showed exactly the same electrophoretic mobility in SDS-PAGE gels, strongly suggesting that a common antigenic protein, hereafter indicated by Mup44 (Muscle protein of 44 kDa), is recognized by these sera.

Association of anti-Mup44 autoantibodies with IBM in myositis sera

To investigate whether anti-Mup44 antibodies are only present in IBM sera or are also found in other myositis sera, sera from DM and PM patients were analyzed by immunoblotting as described above. For these analyses, the sera of the myositis cohort recently characterized by Abdo *et al.* was used [25]. As shown in Table 1, 29% of the IBM sera in this cohort contain autoantibodies to Mup44. Although DM and PM sera were reactive with several antigenic proteins in muscle extracts (Fig. 2 and data not shown), none of the DM and only one of the PM sera appeared to show reactivity with Mup44, indicating a strong specificity for IBM. The relatively high frequency of autoantibodies directed to Mup44 in IBM sera was confirmed in two independent IBM cohorts, in which 14% and 25% of reactive sera were observed, respectively (Table 1). A combination of the data from the three IBM cohorts results in an overall anti-Mup44 reactivity in 23% of IBM sera (21 of 91 serum samples) (Table 2).

Prevalence of autoantibodies to Mup44 in other autoimmune patient sera

To further explore the disease-specificity of the autoantibodies directed to Mup44, sera from patients with several autoimmune diseases, PM-Scl, Scl, RA, SLE and SjS as well as serum from healthy individuals, were analyzed by immunoblotting. As shown in Table 2, none of the healthy controls, PM-Scl, Scl and RA patient sera contained autoantibodies directed to Mup44. In contrast, anti-Mup44 reactivity was detected in 9% of SLE sera and 7% of SjS sera.

Co-occurrence of MAA and anti-Jo-1 with anti-Mup44 autoantibodies

Although in IBM no disease-specific autoantibodies have been described, MAA such as anti-Ro52, anti-Ro60 and anti-La have been reported to be present [10,11,22,23]. To investigate whether the presence

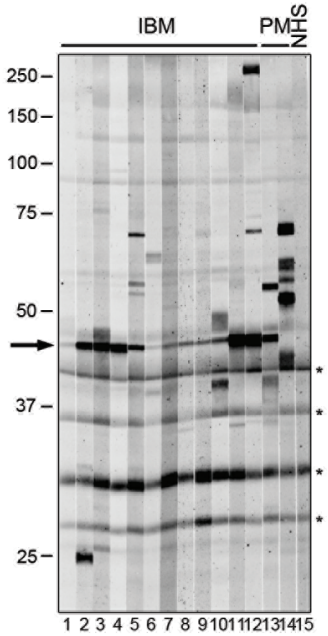

Fig. 2 IBM sera identify a common 44 kDa muscle protein.
Human skeletal muscle extract was separated by SDS-PAGE and blotted to nitrocellulose membranes. The blot strips were incubated with IBM patient sera (lanes 1-12), PM sera (lanes 13-14) and healthy control serum (NHS; lane 15), followed by detection of the bound antibodies by incubation with an IRDye800-labeled anti-human secondary antibody. Bound antibodies were visualized with the Odyssey infrared imaging system. The arrow marks the position of Mup44. The positions of molecular weight markers are indicated on the left; asterisks mark non-specific bands resulting from the reactivity of the secondary (anti-human) antibody with muscle extract proteins.

of these and anti-Jo-1 antibodies correlated with the anti-Mup44 reactivity, available serological data for two of the three IBM cohorts (Hengstman and Badrising cohorts) were compared. Analysis of the co-occurrence revealed no difference in the prevalence of these antibodies in anti-Mup44-positive and anti-Mup44-negative patients (Table 3). Thus, there appears to be no preference for a combined presence of IBM-associated Mup44 and MAA in IBM serum samples.

Discussion

In this study we revealed for the first time the presence of IBM-associated autoantibodies. By using human skeletal muscle proteins as substrates in immunoblotting we were able to identify a 44 kDa muscle autoantigen, named Mup44, which is recognized by 23% of IBM sera, whereas it is hardly or not reactive with other myositis sera. The frequency of anti-Mup44 reactivity in IBM sera is indeed relatively high when compared with other autoantibody reactivities in myositis. It is similar to that of the most frequently detected MSA anti-Jo-1, which is targeted by 20%-30% of PM and DM patients [7,8,10,23]. The specific association of anti-Mup44 with IBM in comparison with PM and DM implies that these Mup44 autoantibodies may be used as an IBM-biomarker in myositis patients.

Although control groups of autoimmune sera from PM-Scl, Scl and RA patients, as well as sera from healthy individuals showed no reactivity with Mup44, some SjS and SLE patient sera did recognize the Mup44 antigen (7% and 9%, respectively). On the other hand, the possibility of (developing) overlap syndromes exists as well. It has been reported that SjS can be found in combination with IBM [22,29,30]. The onset of SjS mostly precedes the onset of IBM, raising the possibility that the two anti-Mup44 positive SjS patients in our study may develop or have developed IBM as well. Similar cases of SLE and IBM overlap have also been reported [30,31], which may explain why some SLE patients contain autoantibodies to Mup44.

We cannot exclude the possibility that anti-Mup44 autoantibodies may rather be myositis-associated than myositis-specific. Interestingly, while myositis-specific Jo-1 autoantibodies are common in DM and PM and appeared to be negatively associated with IBM [32], anti-Mup44 antibodies showed an opposite distribution. Also, MAA found so far can all be detected in both PM and DM patients and often also in IBM [10,11,13,23], which is in contrast to the distribution of the anti-Mup44 autoantibodies.

Previously, it was common practice to use cultured cell lines such as HeLa cells for the identification of autoantigens in autoimmune diseases (for examples see [33-35]). As a result of the apparent absence of Mup44 expression in such cell lines the patient antibodies to this protein may have escaped detection so far. Interestingly, Dalakas *et al.* noted in 1997 the recognition by IBM patient sera of various human muscle antigens ranging in size from 35 to 145 kDa, an observation which was, however, not explored in more detail [36]. Notably, most of the previously identified autoantibodies in myositis target ubiquitously expressed molecules [13]. The use of cultured cell lines as antigen source may have contributed to the idea that autoantibodies are less frequently produced in IBM than for instance in PM and DM [7,10].

The presence of specific autoantibodies in PM and DM is correlated with specific clinical phenotypes, e.g. anti-Jo-1 is a marker for the anti-synthetase syndrome, while anti-SRP antibodies are markers for a necrotising myopathy [7,37]. It will therefore be very interesting to compare the clinical symptoms and

prognosis of IBM patients with the presence of anti-Mup44 autoantibodies in their sera to determine the value of these novel autoantibodies as biomarkers for specific phenotypes. In a preliminary investigation with a limited number of patients we found no obvious differences in clinical features, but this study has to be extended to obtain conclusive results.

The molecular identity of the Mup44 antigen is not resolved yet and is currently under investigation. The identification of the Mup44 corresponding gene, its expression and the analysis of its immunoreactivity will further clarify the disease-specificity of the anti-Mup44 antibodies. The molecular identity of Mup44 will also shed light on the target(s) of the immune system in IBM and may aid the understanding of IBM pathophysiology.

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Chapter 8

General discussion

Polymyositis (PM), dermatomyositis (DM) and inclusion-body myositis (IBM) form the main disorders in the group of idiopathic inflammatory myopathies, which are seen with a frequency of 1 to 6 per 100,000 individuals. Diagnostics and efficient treatment are improving due to the development of techniques with enhanced specificity and sensitivity, although the etiology remains elusive. The presence of autoantibodies in patients' sera and inflammatory loci in patients' muscle biopsies emphasize the immunological nature of myositis. To elucidate whether these phenomena contribute to the development of the disease, would be of great importance for future treatment strategies for this relatively unknown disease. Post-translational modification of biomolecules induced by a number of endo- or exogenous factors are known to be associated with functional effects as well as the loss of immunological tolerance and the development of autoantibodies against neo-epitopes in several autoimmune diseases. The research described in this thesis was focused on the autoantigenic and functional effects of post-translational modifications of the Jo-1 (or histidyl-tRNA synthetase) protein, which is amongst the most frequently targeted autoantigenic proteins by autoantibodies in PM/DM patients.

Myositis classification facilitates disease prognosis and treatment

As described in Chapter 2, more advanced and less invasive imaging techniques will further improve the differentiation between myositis subtypes. However, to date the diagnosis cannot solely be based on non-invasive imaging procedures yet, which stresses the importance of specific serum biomarkers. In recent years several novel myositis-specific autoantibodies (MSA) have been described, which can be correlated to distinct clinical phenotypes and may predict disease progression and/or prognosis (for an overview see Chapter 2). Nevertheless, at least 10% of myositis patients are completely devoid of known autoantibodies, which might be due to different pathological mechanisms underlying the disease and/or technical limitations of the currently used detection methods. This emphasizes the need of multiplex assays that allow the simultaneous detection of multiple autoantibody reactivities and can lead to the identification of biomarker (autoantibody) profiles. Moreover, the development of protein arrays will facilitate the screening of tissue specific proteomes (described in the next paragraphs). Although these techniques show great potential, further investigation is needed to improve the current sensitivity and specificity of these approaches as well as their implementation in the clinic. The currently described autoantibody profiles are used to subtype the different myositis entities but may also reveal whether different mechanisms underlie the development of clinically distinct symptoms and autoantibodies. In addition, these patient specific autoantibody profiles may be applicable to develop personalized therapeutic programs that stop or inhibit disease progression. The subclassification of myositis patients already proved its significance for treatment, since in general IBM patients do not respond to immunosuppressive drugs and therefore their treatment can be adapted early in the disease process, which eliminates unnecessary negative side effects and improves the quality of life.

Tilting the balance from immunological tolerance to autoimmunity

Studies on autoimmune disorders using familial history and mono- and dizygotic twins suggest that the development of autoimmune diseases generally depends on a combination of genetic predispositions and environmental factors. The concordance rates indicate that environmental agents account for approximately 70% of the risk of developing an autoimmune disease whereas the remaining 30% are of genetic influence. Several genetic risk or protective factors have already been described for myositis and a number of predispositions that correlate with autoantibodies, human leukocyte antigen (HLA), or latitudinal differences are summarized in Table 1. Many of these genes regulate responses to environmental agents such as HLA and immunoglobulin gamma heavy chain genes. A number of polymorphic loci in these genes are found to be risk factors for the major myositis types (Table 1). In addition, in the TNFα gene that encodes the proinflammatory tumor necrosis factor α protein, homozygous polymorphisms are found at position 238A and 308A that are associated with distinct clinical manifestions or increased TNFα levels. A missense single nucleotide polymorphism is reported in the minor T allele of protein tyrosine phosphatase, non-receptor type 22 (PTPN22), which causes an amino acid substition (R620W) that is believed to be a risk factor for idiopathic inflammatory myopathies in general. A latitudinal gradient in the frequency of the R620W substitution is reported as well. For further reading about the genetic implications in myositis, the reader is referred to recent reviews by Chinoy *et al.*, O'Hanlon and Miller, and Rider and Miller [1-3]. Hormones as well as a number of environmental

Table 1. Genetic factors that are associated with myositis

Genetic polymorphism[a]	Phenotype	References
HLA DRB1*0301[b]	PM[b], DM, JDM, IBM, anti-synthetase	[1,2,61]
HLA DRB1*0701	Anti-Mi-2, anti-Jo-1	[2,61]
HLA DRB1*0302	Anti-Mi-2	[61]
HLA DQA*0301	JDM, anti-p155	[2,62]
TNFα-238A	JDM	[63]
TNFα-308A	PM, DM, JDM, anti-Jo-1	[1,63,64]
Gm 3 23 5	PM, DM, JDM, IBM, anti-synthetase	[64]
Gm 13	JDM	[64]
PTPN22 R620W	PM, JDM, anti-Jo-1, anti-p155/140, latitudinal gradient in Europe	[65,66]

[a] Polymorphisms described above have not been subdivided into protective or risk factors nor linked to ethnic differences. [b] Abbreviations: *HLA*: human leukocyte antigen, *PM*: polymyositis, *DM*: dermatomyositis, *JDM*: juvenile DM, *IBM*: inclusion body myositis, *TNF*α: tumor necrotsis factor α, *Gm*: immunoglobulin gamma heavy chain, *PTPN22*: protein tyrosine phosphatase, nonreceptor type 22.

factors such as toxins or viruses are suggested to increase the risk for the development of autoimmunity in genetically susceptible individuals.

Several hypotheses for the mechanisms involved in the loss of immunological tolerance have been proposed, three of which will be discussed briefly in this paragraph. First, the so-called molecular mimicry model suggests that autoantibodies in patients initially are generated against epitopes present on bacteria or viruses, and can crossreact with self proteins (or self antigens). Several autoantibodies detected in systemic lupus erythematosus (SLE) patients, including anti-Sm-B/B', anti-Sm-D, and anti-Ro60 have been shown to crossreact with the Epstein-Barr virus nuclear antigen-1 (EBNA-1) [4]. Since an epitope on Ro60 was reported to be recognized by predisease SLE serum samples, which crossreact with a peptide of EBNA-1, it was suggested that Epstein-Barr virus (EBV) might initiate the development of SLE. In addition, SLE patients appeared to be infected by EBV more frequently than healthy controls, which emphasizes the potential role of infectious pathogens in the loss of immunological tolerance [5]. A similar association has been postulated for the development of other autoimmune disorders including Sjögren's syndrome, rheumatoid arthritis (RA), and myositis [6-8]. A number of cases have been reported in which myositis-like symptoms occur in virus infected individuals, whereas in some myositis patients viral particles or viral mRNA was detected in the disease' main target tissue (skeletal muscle) [6,9,10]. However, no conclusive evidence has been reported yet, although a seasonal onset and studies using animal models endorse viral induction as a potential model to lose immunological tolerance [11-14]. The infectious agents that have been associated with the development of myositis were recently reviewed by Crum-Cianflone [6].

Second, the anti-idiotypic antibody model relies on specific idiotypes, which are characteristic structures within a group of immunoglobulin or T cell receptor molecules and are based on the antigenic binding specificity of the variable region of the antibody or T-cell receptor. These structures are recognized by anti-idiotypic antibodies, which are known to occur in autoimmune diseases [15]. Antibodies with a specific idiotype are believed to trigger a cascade in which the production of anti-idiotypic antibodies is followed by the production of anti-anti-idiotypic antibodies. The resulting network of interacting antibodies has been postulated to be involved in both physiological and pathological processes [16,17].

Finally, the third hypothesis predicts that distinct physiological and pathological circumstances can lead to the post-translational modification (PTM) of a myriad of self antigens, which when presented to the immune system might break immunological tolerance and result in the production of autoantibodies. The studies and hypotheses formulated in this thesis are based on this model. The asset of this hypothesis is that it combines non-immune mediated triggers such as endoplasmatic reticulum stress or hypoxia, with immune mediated mechanisms such as those described above. The following paragraphs will elaborate on the established and putative protein modifications and mechanisms involved in autoimmunity, mainly focusing on myositis.

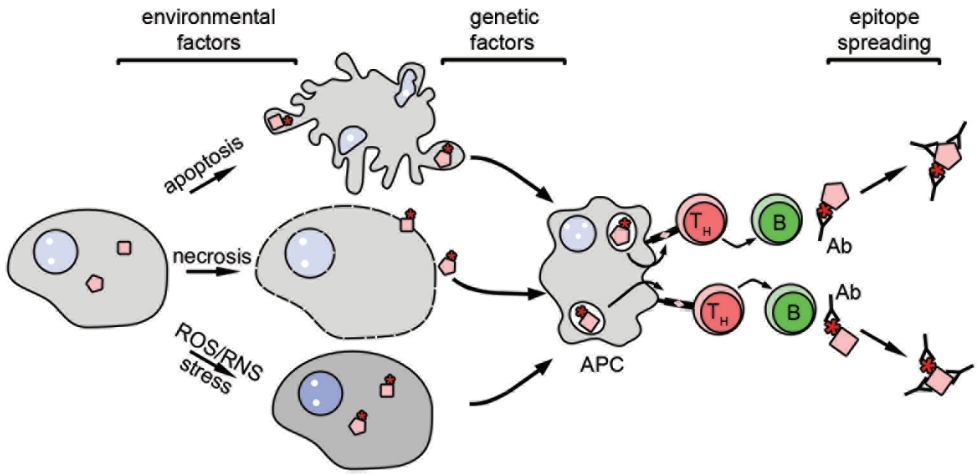

Fig. 1 Model for breaking immunological tolerance by post-translational modifications.
Environmental triggers or tissue injury may induce cell death (e.g. apoptosis or necrosis) or lead to the production of ROS/RNS. Either apoptosis, necrosis and/or ROS/RNS stress can induce modifications (asterisks) of endogenous proteins, which may lead to the formation of neo-epitopes. Antigen presenting cells (APC) present modified peptides and proteins, resulting from cell death or ROS/RNS stress to the immune system in genetically susceptible individuals when clearance mechanisms are defective. This will result in the activation of autoreactive T helper cells (T_H) and initiation of an autoimmune response, including the activation of B cells (B), followed by the production of autoantibodies (Ab). Over time epitope spreading may lead to autoantibodies that recognize the same antigen in its non-modified state or even antibodies to an associated protein.

Are cell death-induced PTMs involved in autoimmunity?

Apoptosis-induced myositis?

A number of mechanisms can lead to cell death (e.g. apoptosis or necrosis), each of which involves specific (non)enzymatic systems. The initiation of these processes can result from several immunological and/or environmental triggers and lead to the post-translational modification of self proteins, which may generate neo-epitopes and break immunological tolerance (Fig. 1). A well known example is found in patients suffering from RA, in which frequently anti-citrullinated protein antibodies can be detected. The peptidylarginine deiminases are believed to play a major role in the disease, since these enzymes catalyze the conversion of peptidylarginine to peptidylcitrulline and are known to be highly active in dying cells. A report that described the immunization of rats with either noncitrullinated or citrullinated rat serum albumin emphasized the role of this modification in the development of citrulline-specific antibodies as well as the ability to break tolerance to self antigens [18]. More recently, Hof and coworkers reported the preferential recognition of an apoptotically cleaved U1-70k protein by autoantibodies in sera of mixed connective tissue disease (MCTD) patients [19]. In individuals suffering from PM and DM inflammatory cell infiltrates, capable of inducing cell death, are often found, but these patients are devoid of loci that show extensive apoptosis in lung and muscle tissues. Studies on the expression of pro- and anti-apoptotic molecules in myositis patients contradict each other [20,21], leaving the question whether apoptotic mechanisms are of importance in the initiation and/or progression of myositis unanswered (see also Chapters 1 and 4). Nevertheless, it is important to note that granzyme B (GrB), which is known to induce caspase-dependent as well as caspase-independent forms of apoptosis, might play a role in myositis [22]. Moreover, the number of GrB substrates expanded in recent years and a GrB susceptible Jo-1 protein has been found specifically in lung tissue, which suggests that tissue specific modifications (discussed in the next paragraph) can underlie different disease entities [23].

Necrosis-induced myositis?

Not much is known about the contribution of necrosis to the development of autoimmune disorders. However, necrotic loci and the infiltration of immune cells into non-necrotic MHC class I expressing muscle fibers have been found in most of the myositis subtypes. These MHC I expressing cells may trigger or continuously stimulate the individual's immune system by presenting cell death induced neo-epitopes to autoreactive lymphocytes. Such necrosis-specific neo-epitopes may be generated by specific cleavages of autoantigenic proteins, which are distinct from the cleavages observed in apoptotic cells [24]. The cleavage products may also be different for the various necrotic triggers and/or signaling pathways

that lead to necrosis [25,26]. In Chapters 3 and 4 compound-specific necrotic modifications on several autoantigenic proteins including Jo-1 are described (Fig. 1). The autoantigens modified upon the exposure of cells to hydrogen peroxide (H_2O_2) were altered well before the appearance of necrotic markers. Many protein modifications fulfill distinct functions during cell death processes such as inhibiting or stimulating protein activity or introducing pro-inflammatory properties. However, since reactive oxygen species (ROS) such as H_2O_2 led to the modification of biomolecules and none of the alternative necrosis-inducing compounds resulted in a similar modification (data not shown), the possibility remains that these modifications are not due to the induction of necrosis, but are rather caused by this type of oxidative stress. Depending on temporal, spatial and conformational differences proteins may become functionally (in) active, and/or highly immunogenic as a result of the same modification. We conclude that H_2O_2-induced oxidative modifications can be involved in the induction of a distinct necrotic pathway and/or can support the generation of neo-epitopes.

Are reactive oxygen/nitrogen species involved in PTM?

In parallel with skeletal muscle fibers (Chapter 1), the lung is suggested to be one of the main target tissues in myositis patients. Interstitial lung disease (ILD) is a manifestation that is strongly associated with myositis patients in which anti-synthetase antibodies are found. The development of ILD is classified based upon a number of causal agents including inhalation of (in)organic substances or infections. In addition, oxidative stress has been suggested to contribute to the epithelial cell damage in interstitial pneumonia [27]. Moreover, since both muscle and lung tissue is exposed to high levels of oxygen, they may provide a source of autoantigens (e.g. Jo-1; Fig. 1) modified by reactive oxygen and nitrogen species (ROS/RNS). Both the infiltrating immune cells and skeletal muscle tissue are able to generate relatively high concentrations of ROS/RNS, which may lead to the modification of biomolecules and induce cell death as well. ROS/RNS-induced protein modifications and/or tissue damage has already been implicated in the pathogenesis of RA, scleroderma and type 1 diabetes [28-30]. A similar role in myositis is supported by the data presented in this thesis. Along with oxidatively modified autoantigens from SLE, scleroderma, and PM/Scl overlap disorders also the major autoantigen in myositis, Jo-1, was found to be modified in cells subjected to oxidative stress. Two of the modified regions coincided with previously identified Jo-1 autoepitopes [31]. It will be interesting to investigate whether Jo-1 is similarly modified in muscles or lungs from myositis patients.

In contrast to the destructive processes of ROS/RNS, a beneficial role for ROS resulting from the NADPH oxidase (NOX2) complex in the regulation of autoreactive T lymphocytes and the development of collagen-induced arthritis has been suggested [32,33]. The dual role of ROS/RNS (protective and destructive) introduces another level of complexity to the biological effects of ROS/RNS. Indeed, protective effects, as a result of ROS, might be observed in myositis patients, since exercise programs report beneficial effects on disease progression and on the number of inflammatory infiltrates [34]. The controlled increase in ROS production, as a result of exercise, might exert protective effects in the local environment of skeletal muscles. However, additional studies that compare local ROS concentrations before and after exercise have yet to be performed.

The distinct nature of RNS reactive groups and the life span of reactive molecules compared to ROS may lead to unique biomolecular modifications. Indeed, distinct RNS induced modifications in human and mouse models have been described to contribute to the pathogenesis of autoimmune diseases (e.g. SLE and RA) [35-37]. The ROS/RNS-induced Jo-1 modification documented in Chapter 6, the reduction-resistant covalent dimerization of Jo-1 involving the C-terminal cysteines, may likewise play a role in the pathogenesis of myositis. The results described in this chapter strongly suggest that peroxynitrite reaction products or radicals, which are able to react with peroxynitrite, are involved in the formation of the yet uncharacterized reduction-resistant Jo-1 dimer crosslink. Follow-up studies using precipitation and mass spectrometry-based identification techniques are needed to elucidate the chemical identity of this protein crosslink.

Does the autoimmune response in myositis target tissue-specific antigens?

In contrast to the tissue-specific autoimmune diseases, most autoantigenic targets of systemic autoimmune diseases are ubiquitously expressed and can be distributed over different subcellular compartments. Their broad expression patterns suggest that these (modified) autoantigens may elicit an autoimmune response whenever neo-epitopes are presented to the immune system. However, there appears to be cell and/or tissue type specificity in

the modification and presentation of autoantigens. Studies of Ulanet *et al.* and of Levine *et al.* provide evidence for the cell type specific cleavage of the B23 and Jo-1 autoantigens, respectively, by GrB [23,38]. They describe a conformation of the autoantigen that renders the protein susceptible to GrB cleavage, which may only occur in certain tissues/cells. The presence of the cleavable Jo-1 protein in the lung may suggest that the initiation and propagation of myositis occurs in lung tissue and spreads to skeletal muscle. These data also suggest that the formation of neo-epitopes on autoantigenic proteins not only depends on the compound or enzyme that is responsible for inducing the modification, but also on the cell type. As a consequence, a number of autoantibodies may have escaped detection, because cultured cells were used to identify and characterize them. In this respect, it seems worthwhile to use material from tissues that are most strongly affected in autoimmune diseases to study disease-specific autoantibodies and their targets. The data presented in Chapter 7 support this idea and provide evidence for the first IBM-specific autoantibodies, which can only be detected by using antigens from skeletal muscle tissue. However, one has to bear in mind that not always the most strongly affected tissue may be the source of the autoantigen that elicits the initial immune response. It will be interesting to study the autoantibody profiles of PM/DM and IBM patients using skeletal muscle as well as lung tissue.

Can autoantibodies aid the diagnosis of myositis?

In general autoimmune patients enter the clinic when the first symptoms pass a certain threshold, which, however, can be years after the initial onset of the disease. Reaching a diagnosis may take several months. In the pre-disease phase epitope spreading may occur and lead to the detection of autoantibody reactivities that are directed against multiple epitopes of one or more autoantigens [39,40]. This process may result in the disappearance of autoantibodies that are reactive with the initially triggering epitope. Therefore it is very important to collect serum samples from the time patients enter the clinic and continue with this during subsequent visits. Such longitudinal serum collections can give insight in how the reactivity to a particular autoantigen develops during the course of the disease, as described for U1-70k by Hof and collaborators [19]. Studies in which serum samples of individuals suffering from RA or SLE, which were collected before the first clinical symptoms appeared (pre-disease samples), were analyzed allowed the detection of one or more epitope specificities. In some cases, the corresponding antibodies could be detected in samples taken many years before the first visit to the clinic, which emphasizes the predictive value of such autoantibody subsets [40-44].

To date extensive information on myositis-specific autoantibodies preceding the disease is still lacking. However, in one case anti-Jo-1 antibodies were found in a patient before muscle disease became manifest [45]. Increasing evidence shows that autoantibodies can be very helpful for the subclassification of myositis patients, the prediction of disease progression, and the responsiveness to treatment [46-49]. Several correlations between autoantibodies and clinical features have been described. Interstitial lung disease is frequently observed in patients with myositis and is strongly associated with the production of aminoacyl-tRNA synthetase autoantibodies [50,51]. The anti-p155/p140 autoantibody has recently been described as one of the most common autoantibodies in cancer-associated DM [52,53].

So far, only few studies report on post-translational modifications of the Jo-1 autoantigen [23,54] and autoantibodies specifically reactive with a modified Jo-1 protein have not been found. Anti-Jo-1 antibodies are known to undergo affinity maturation and class switching [45]. Since the anti-Jo-1 positive patient sera used in the experiments described in Chapter 4 and 6 were obtained from established myositis patients, epitope spreading may have occurred. This emphasizes the importance to investigate pre-disease sera for the occurrence of anti-ROS/RNS-modified Jo-1 autoantibodies.

Does ROS/RNS-induced crosslinking functionally affect proteins?

As described above, post-translational modifications can generate neo-epitopes, which may result in the loss of immunological tolerance and the initiation of autoimmunity. However, radical-induced modifications are also known to affect functional activities. The fluctuations of the intracellular redox status affect many proteins such as aminoacyl-tRNA synthetases, transcription factors, and protein kinases and can provide a regulatory mechanism for metabolic signaling and transcriptional processes via the reversible modification of proteins involved [55-58]. For instance, gene expression in contractile muscle cells is partially regulated by NF-κB, which is activated upon the release of IκB. In addition to the phosphorylation dependent release of IκB, the NF-κB/IκB complex can also dissociate upon ROS stimulation [59]. The influence of the redox status

on factors involved in translation is illustrated by the oxidation of cysteines in prokaryotic glutamyl-tRNA synthetase, which results in impaired $tRNA^{Glu}$ aminoacylation *in vitro* [60]. Two isoforms of the tryptophanyl-tRNA synthetase (TrpRS) exist in human cells, the major full-length form (TrpRS), and a truncated form that lacks the N-terminal extension (mini-TrpRS). Both forms are able to aminoacylate $tRNA^{Trp}$, but Wakasugi and coworkers observed an increased aminoacylation activity of mini-TrpRS but not of TrpRS as a result of binding to oxidized GAPDH [58]. These studies emphasize the role of oxidative stress-induced regulation of cellular processes. Increasing evidence suggests that thiols can play a key role in the regulation of redox-dependent systems. This may also be the case for histidyl-tRNA synthetase (Jo-1), because elevated aminoacylation activity was observed under conditions that led to oxidative changes of the thiols close to the C-terminus (Chapters 4 and 5). Since the Jo-1 protein is known to function as a homodimeric protein *in vivo*, the disulfide bonds involved in crosslinking the Jo-1 dimer (Chapter 5) may stabilize the dimer and alter the balance between dimeric and monomeric Jo-1, which might lead to an increased aminoacylation activity (Fig. 2). It would be interesting to investigate whether similar crosslinking events occur in other di- or multimeric aminoacyl-tRNA synthetases. The increased activity of crosslinked Jo-1, and other tRNA synthetases or factors involved in protein synthesis, may temporarily elevate protein synthesis in order to restore the redox balance.

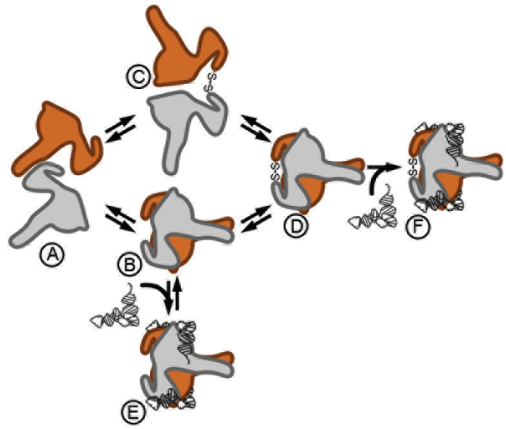

Fig. 2 Dimerization and crosslinking of Jo-1.
Under normal physiological circumstances Jo-1 resides either as a monomer (A) or as a homodimer (B) in the cytoplasm. Under oxidative conditions crosslinked dimers can form, e.g. by disulfide bonds between their C-terminal cysteine side chains (C, D). The covalent bond(s) in the Jo-1 dimer may shift the equilibrium between B and D to D. Both B and D will associate with histidine and $tRNA^{His}$ and will catalyze the aminoacylation of $tRNA^{His}$ (E, F).

Concluding remarks and perspectives

This thesis provides an overview of myositis-specific autoantibodies and their detection methods, and discusses their significance for disease classification and prognosis. In addition, the research described contributed to understanding the role of oxidative/nitrosative stress-induced protein modifications in the development of autoantibodies. Especially the functional and autoantigenic properties of the myositis-specific autoantigen Jo-1 are thoroughly investigated. The elucidation of disulfide-dependent and independent Jo-1 homodimer crosslinks, the apparent oxidative stress-regulated tRNA-aminoacylation activity as well as the structural Jo-1 model open the door to new research questions. Are oxidative/nitrosative stress-modified proteins present in affected tissues of autoimmune diseases? Do autoantibodies to oxidative/nitrosative stress-induced modifications exist and, if so, develop early in disease? Why is the tRNA-aminoacylation activity of Jo-1 upregulated under oxidative stress conditions? Are the C-terminal cysteines in the Jo-1 protein involved in an intracellular redox-regulated mechanism? What is the chemical nature of the cysteine-dependent, reduction-resistant Jo-1 crosslink? Does the structural Jo-1 model correspond to the endogenous human Jo-1 dimer? The data described in this thesis may facilitate follow-up studies to elucidate the questions above and to obtain further insight in the role of Jo-1 in (patho)physiological processes.

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Summary

During the last decades the number of patients suffering from an autoimmune disease has gradually increased. Currently, these disorders affect approximately 2-3% of the world population. One of the most pronounced molecular features is the presence of autoantibodies in the sera of patients. Whether these autoantibodies contribute to the development of the disease or are merely an epiphenomenon remains unclear. In recent years, however, some of the autoantibodies have been reported to be not only disease-specific and useful as diagnostic biomarkers, but also predictors of disease progression. For instance, in patients with rheumatoid arthritis citrullinated proteins are targeted by autoantibodies, which can be found years prior to the development of clinical symptoms. Additionally, citrullination-specific autoantibodies are almost exclusively found in these patients and their presence predicts the severity of disease progression. Therefore they can serve as serological and prognostic markers. Besides the physiological process of citrullination autoantibody targets have been suggested to become citrullinated in the extracellular environment by activated peptidylarginine deaminase (PAD) enzymes, which are released by dying cells. The presentation of the aberrantly modified proteins to the immune system is believed to break immunological tolerance and result in the development of autoimmunity. The post-translational modifications of proteins as a result of exogenous or endogenous triggers that lead to the loss of immunological tolerance is regarded as a potential mechanism in the development of autoimmune diseases.

Idiopathic inflammatory myopathies, also indicated with myositis, represent a group of autoimmune disorders in which mainly skeletal muscles are affected. Patients suffering from myositis are characterized by skeletal muscle weakness, atrophy and inflammatory features. Because of distinct clinical, serological and histological phenotypes the idiopathic inflammatory myopathies are most commonly diagnosed as polymyositis (PM), dermatomyositis (DM) and inclusion-body myositis (IBM), for which specific criteria have been defined. The sera of myositis patients often contain disease-specific autoantibodies, and inflammatory cell infiltrates can be detected in patients' skeletal muscle tissue. All of these characteristics are currently being used for diagnostic and prognostic purposes, but in spite of intensive study no clear causative agents have been identified yet.

Based upon the hypothesis that the unusual post-translational modifications of autoantigens may elicit a primary immune response in the early stages of the development of autoimmunity and the fact that skeletal muscles are often oxidatively stressed, we decided to investigate the effects of oxidative stress on one of the main targets of the immune system in myositis, the Jo-1 autoantigen. In the studies described in this thesis a number of reactive oxygen and nitrogen species (ROS/RNS)-induced modifications of Jo-1, which is the histidyl-tRNA synthetase (HisRS), were identified and characterized. In addition, the importance of using autoimmune-associated tissues in the detection of novel myositis autoantibodies was explored. The goal was to obtain insight in autoantibody targets and the role of ROS/RNS-induced modifications in the reactivity of myositis-associated autoantibodies.

Chapter 1 contains a comprehensive overview of our current knowlegde of cell death- and cellular stress-associated processes that are believed to be involved in the development of myositis. An overview of the targets of myositis-specific autoantibodies and their biochemical features is presented in Chapter 2. In this chapter also the most commonly applied and novel autoantibody detection methods are discussed, as well as the importance of autoantibodies in the diagnosis of myositis, prediction/monitoring of disease progression and treatment.

It was investigated whether ROS can induce protein modifications that may be involved in the generation of neo-epitopes. Chapters 3 and 4 describe ROS-induced modifications in a number of autoantibody targets associated with different autoimmune disorders, including systemic lupus erythematosus, systemic sclerosis, and myositis. A similarly retarded migration in SDS-PAGE was observed for several autoantigens in extracts of H_2O_2-treated Jurkat cells, in oxidized cell lysates, as well as for the corresponding recombinant proteins (recU1A and recHisRS) that were expressed in bacteria and oxidized in vitro. Further analyses of the modified HisRS demonstrated that several methionines and a single tryptophan residue becomes oxidatively modified. Several experimental approaches to investigate the presence of autoantibodies to the oxidatively modified HisRS so far did not lead to the detection of autoantibodies in the tested myositis patient sera.

In addition to the slight migratory retardation of HisRS in SDS-PAGE upon oxidative stress, also a more slowly migrating species at a position corresponding

to twice the molecular weight of HisRS was observed. Reduction of the protein prior to gel electrophoresis appeared to eliminate the appearance of this putative HisRS dimer (Chapter 5). Further analyses showed that indeed the formation of one or two disulfide bond(s) was responsible for the generation of this HisRS isoform. Thus, upon hydrogen peroxide-induced stress the HisRS protein is covalently crosslinked by the generation of disulfide bonds, which are dependent on two cysteine residues in the C-terminal tail of the HisRS subunits. Since such modifications might induce structural and functional changes the HisRS activity was analyzed in an in vitro aminoacylation assay. The oxidative stress-induced HisRS modifications did not abrogate its $tRNA^{His}$ aminoacylation activity, but instead slightly enhanced this activity.

In addition to oxidative stress induced by the exposure to hydrogen peroxide, Jurkat cells were also treated with SIN-1, which is known to lead to the production of both ROS and RNS. In Chapter 6 we report the formation of another covalent dimer of the HisRS protein in cells treated with SIN-1. In contrast to the previously detected dimer, this isoform of HisRS appeared to be resistant to reducing agents, indicative of another chemical bond. Nevertheless, mutagenesis studies showed that the same cysteines near the C-terminus of HisRS are involved in the induction of this HisRS dimer. The exact nature of the bonds in this homodimer remains to be determined. The HisRS modifications induced by SIN-1 neither affected its catalytic activity nor its immunoreactivity with antibodies in myositis patient sera.

As mentioned in Chapter 1, the elucidation of autoantibody profiles are becoming more important in recent years, because of their potentially high diagnostic and prognostic relevance. In studies that elucidate novel autoantibody reactivities often material from cultured cells has been used, in spite of the availability of tissues that are most strongly affected in the disease. The potential of detecting novel autoantibodies in myositis by using skeletal muscle material was addressed in the work described in Chapter 7. Although this did not lead to the identification of new reactivities associated with poly- and/or dermatomyositis, a novel autoantibody reactivity was found for inclusion-body myositis. Inclusion-body myositis was regarded to be a primarily degenerative disease with secondary immune components and the detection of certain autoantibodies has been suggested to exclude the diagnosis of IBM. However, using human skeletal muscle tissue extracts autoantibodies to a protein of 44 kDa (Mup44) were detected in 23% of IBM patient sera. Importantly, anti-Mup44 reactivity was specific for IBM and almost not detected in sera from PM and DM patients. Anti-Mup44 represents the first disease-specific autoantibody marker for IBM.

In Chapter 8 the findings described in this thesis are discussed in a broader context. The importance of determining genetic and environmental risk or protective factors in myositis patients as well as how autoantibodies can contribute to diagnosis or predict disease development are further elaborated. The possibility that the environmental factors induce cell death and that the post-translational modifications occurring under these conditions play a role in breaking immunological tolerance to self-components is discussed. In this context also tissue-specific aspects may be important. More specifically, the implications of the dimerization and crosslinking of the histidyl-tRNA synthetase as a result of the formation of covalent bonds, as observed in oxidatively stressed cells, are addressed.

Samenvatting

Gedurende de laatste decennia is het aantal patiënten die aan auto-immuunziekten lijden gestaag gestegen. Momenteel lijdt circa 2-3% van de wereldbevolking aan dit ziektebeeld. Eén van de meest uitgesproken moleculaire kenmerken is de aanwezigheid van autoantilichamen in sera van patiënten. Het is nog onduidelijk of deze autoantilichamen aan de ontwikkeling van de ziekte bijdragen of niet meer als een bijverschijnsel van het ziektebeeld zijn. Maar in de afgelopen jaren is voor sommige autoantilichamen niet alleen beschreven dat ze ziekte-specifiek of bruikbaar als diagnostische biomarkers zijn, maar is duidelijk geworden dat ze het ziekteverloop kunnen verspellen. Bijvoorbeeld in patiënten met reumatoïde artritis kunnen al jaren voor de ontwikkeling van klinische symptomen, autoantilichamen worden gevonden welke gecitrullineerde eiwitten herkennen. Tevens worden citrullinatie-specifieke autoantilichamen bijna uitsluitend in deze patiënten gevonden en voorspeld hun aanwezigheid de hevigheid van het ziekteverloop. Hierdoor kunnen ze als serologische en prognostische markers gebruikt worden. Naast het fysiologische proces van citrullinatie wordt gedacht dat peptidylarginine deaminase (PAD) enzymen, welke door stervende cellen uitgescheiden worden, in het extracellulaire milieu autoantilichaam doelwitten citrullineren. Men denkt dat de presentatie van ongebruikelijk gemodificeerde eiwitten aan het immuun systeem leidt tot het doorbreken van de immunologische tolerantie en resulteert in de ontwikkeling van autoimmuniteit. De post-translationele modificatie van eiwitten, als gevolg van exogene of endogene schakelaars, die leiden tot het verlies van immunologische tolerantie, wordt als mogelijk mechanisme voor de ontwikkeling van auto-immuunziekten gezien.

Idiopathische inflammatoire myopathieën welke ook wel myositis genoemd worden, zijn een groep auto-immuun stoornissen waar voornamelijk de skeletspieren zijn aangetast. Patiënten met myositis lijden aan een skeletspier zwakte, atrofie en hebben ontstekingskenmerken. Door opmerkelijke klinische, serologische en histologische fenotypen worden de idiopathische inflammatoire myopathieën over het algemeen als polymyositis (PM), dermatomyositis (DM) en inclusion-body myositis (IBM) gediagnosticeerd, waarvoor speciale criteria zijn vastgesteld. De sera van myositis patiënten bevatten vaak ziekte-specifieke autoantilichamen en ontstekingshaarden, bestaande uit immuun cellen, en kunnen vaak in het skelet spierweefsel van patiënten worden aangetoond. Al deze kenmerken worden tegenwoordig voor diagnostische en prognostische doeleinden gebruikt. Maar ondanks intensief onderzoek is er nog geen oorzaak van de ziekte geïdentificeerd.

Van de hypothese uitgaande dat ongebruikelijke post-translationele modificaties van autoantigenen, een primaire immuun respons tijdens de eerste stappen van de ziekte ontwikkeling te weeg kunnen brengen en het feit dat skeletspieren vaak oxidatief gestresst zijn, hebben we besloten de effecten van oxidatieve stress bij het Jo-1 autoantigeen te bestuderen, welke een van de hoofddoelwitten van het immuun systeem in myositis is. De studies in dit proefschrift beschrijven en karakteriseren een aantal reactieve zuurstof en stikstof species (ROS/RNS) geïnduceerde modificaties van Jo-1, ofwel histidyl-tRNA synthetase (HisRS). Tevens wordt het nut van autoimmuun-geassocieerde weefsels voor de detectie van nieuwe myositis autoantilichamen onderzocht. Het doel was om inzicht in autoantilichaam doelwitten te krijgen en welke rol ROS/RNS geïnduceerde modificaties in de reactiviteit van myositis geassocieerde autoantilichamen spelen.

In hoofdstuk 1 wordt een uitgebreide beschrijving gegeven van onze huidige kennis over celdood en celstress geassocieerde processen, waarvan gedacht wordt dat ze betrokken zijn bij de ontwikkeling van myositis. hoofdstuk 2 presenteert een overzicht van de myositis-specifieke autoantilichaam doelwitten en hun biochemische kenmerken. Tevens worden de meest gebruikte en nieuwe autoantilichaam detectie methoden en de relevantie van autoantilichamen in de diagnose van myositis, en voorspelling/monitoring van ziektevoortgang en behandeling in dit hoofdstuk bediscussieerd.

Er is onderzocht of ROS eiwit modificaties te weeg kunnen brengen die mogelijk betrokken zijn bij de vorming van neo-epitopen. Hoofdstukken 3 en 4 beschrijven ROS-geinduceerde modificaties in een aantal autoantilichaam doelwitten, welke geassocieerd zijn met verschillende auto-immuunstoornissen, waaronder systemic lupus erythematosus, systemic sclerosis, en myositis. Voor verschillende autoantigenen kon in extracten van H_2O_2 behandelde Jurkat cellen, geoxideerd cellysaat en bijbehorende recombinant eiwitten (recU1A en recHisRS), welke in bacteriën tot expressie gebracht waren en *in vitro* geoxideerd, een vergelijkbaar vertraagde migratie in SDS-PAGE worden vastgesteld. Verdere analyse van het gemodificeerde

HisRS wees uit dat verschillende methionines en een enkel tryptofaan residue oxidatief gemodificeerd was. Met behulp van verschillende experimentele methodes is onderzocht of autoantilichamen tegen de oxidatief gemodificeerd HisRS aanwezig waren, maar tot dus ver zijn er geen autoantilichamen in de geteste myositis patiënt sera gevonden.

Bovenop de vertraagde migratie van HisRS in SDS-PAGE onder oxidatieve stress, is een nog langzamer migrerend product zichtbaar, welke overeenkomt met twee maal het moleculair gewicht van HisRS. Door het eiwit voor gel electroforese te reduceren is het mogelijk om de vermoedelijke HisRS dimeer te laten verdwijnen (hoofdstuk 5). Verdere analyses lieten zien dat inderdaad de vorming van één of twee disulfide bindingen verantwoordelijk waren voor de vorming van deze HisRS isovorm. Hieruit volgt dat onder invloed van waterstof peroxide geïnduceerde stress de HisRS eiwitten een covalente binding aangaan door de vorming van disulfide bruggen, welke afhankelijk zijn van twee cysteine residuen in de C-terminale staart van de HisRS subunits. Omdat zulke modificaties mogelijk structurele en functionele veranderingen te weeg brengen, is de HisRS activiteit geanalyseerd met behulp van een *in vitro* aminoacylatie assay. De oxidatieve stress geïnduceerde HisRS modificaties heffen de $tRNA^{His}$ aminoacylerings activiteit niet op, maar lijken de activiteit licht te verhogen.

Jurkat cellen werden niet alleen blootgesteld aan oxidatieve stress, als gevolg van waterstof peroxide, maar ook behandeld met SIN-1 wat leidt tot de productie van ROS en RNS. In hoofdstuk 6 beschrijven we de vorming van nog een covalente dimeer van het HisRS eiwit welke word gevormd in cellen die behandeld zijn met SIN-1. In tegenstelling tot de hiervoor beschreven dimeer, blijkt deze isovorm van HisRS resistent tegen reducerende omstandigheden te zijn, wat de aanwezigheid van een andere chemische binding aan geeft. Daarentegen wijzen mutagenese studies erop dat dezelfde cysteines aan de C-terminus van HisRS betrokken zijn bij de vorming van deze HisRS dimeer. De exacte aard van de binding in deze homodimeer moet nog worden opgehelderd. De SIN-1 geïnduceerde HisRS modificaties hebben noch een effect op de katalytische activiteit noch op de immunoreactiviteit van antilichamen in myositis patiënten sera.

Zoals in hoofdstuk 1 vermeld, wordt door hun potentiële diagnostische en prognostische relevantie het ophelderen van autoantilichaam profielen in de laatste jaren steeds belangrijker. In de huidige studies, die nieuwe autoantilichaam doelwitten onderzoeken, wordt vaak materiaal uit cel culturen gebruikt, ondanks de beschikbaarheid van weefsels die het meest aangedaan zijn in een ziektebeeld. De mogelijkheid om nieuwe autoantilichamen in myositis te detecteren, door gebruik te maken van skeletspier materiaal, wordt in hoofdstuk 7 beschreven. Hoewel er geen nieuwe autoantilichamen voor polymyositis en/of dermatomyositis geïdentificeerd werden, is een nieuw autoantilichaam voor inclusion-body myositis gevonden. Inclusion-body myositis werd primair als degeneratieve ziekte gezien met secondaire immunologische aspecten, waarbij de detectie van bepaalde autoantilichamen de IBM diagnose leken uit te sluiten. Maar met behulp van humaan skeletspierweefsel extracten zijn autoantilichamen in 23% van IBM patiënten sera ontdekt, die reactief zijn met een eiwit van 44 kDa (Mup44). Belangrijk is dat de anti-Mup44 reactiviteit specifiek was voor IBM en nagenoeg niet detecteerbaar in sera van PM en DM patiënten. Anti-Mup44 is de eerste ziekte specifieke autoantilichaam marker voor IBM.

Hoofdstuk 8 bediscussieerd de bevindingen die in dit proefschrift beschreven zijn in een bredere context. Het belang van gedefinieerde genetische en omgevingsfactoren in myositis patiënten, die een risico vormen of juist beschermend werken, als wel hoe autoantilichamen kunnen bijdragen aan de diagnose of het voorspellen van de ziekte ontwikkeling, worden verder uitgelegd. De mogelijkheid dat omgevingsfactoren celdood kunnen induceren en dat post-translationele modificaties tijdens deze condities een rol spelen in het verlies van immunologische tolerantie tegen self-componenten wordt bediscussieerd. Hierbij is het ook mogelijk dat weefsel-specifieke aspecten belangrijk zijn. Ofwel de dimerizatie en crosslinking van het histidyl-tRNA synthetase als gevolg van de vorming van covalente bindingen, zoals in oxidatief gestresste cellen, wordt beschreven.

Dankwoord

Bijna de laatste regels van dit proefschrift en zoals in de vorige bladzijdes valt te lezen, is dit niet tot stand gekomen door een enkeling maar hebben een hoop mensen hieraan bijgedragen. Ik wil hierbij graag iedereen bedanken die op wat voor manier dan ook een bijdrage hieraan heeft geleverd. Hierbij gaat naar enkelen mijn bijzondere dank uit.

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Curriculum vitae

Sander van Dooren is op 17 juli 1980 geboren te Tegelen. In 1996 haalde hij zijn MAVO diploma aan het Dendron College te Horst. Waarna hij aan de Fontys Middelbaar Laboratorium Opleiding (MLO) in Blerick begon. In 2000 behaalde hij hier, met specialisatie medische microbiologie, zijn diploma. In hetzelfde jaar begon hij het verkorte traject aan de Hoger Laboratorium Opleiding (HLO) met specialisatie bio(proces)technologie, aan de Hogeschool Arnhem & Nijmegen. Na het behalen van het diploma in 2003 begon hij aan een verkorte universitaire opleiding medische biologie aan de Radboud Universiteit Nijmegen. Waar in 2006 hij de Master of Science bul in ontvangst heeft genomen.

Vanaf oktober 2006 tot juni 2011 heeft hij als promovendus op de afdeling Biomoleculaire Chemie van de Radboud Universiteit Nijmegen gewerkt. Onder supervisie van Prof. dr. W.J. van Venrooij en Prof. dr. G.J.M. Pruijn heeft hij het onderzoek verricht dat beschreven is in dit proefschrift. Tijdens deze periode heeft hij deelgenomen aan verschillende internationale meetings en congressen die binnen en buiten het Autocure consortium georganiseerd zijn.

Midden 2011 is hij verhuisd naar Bonn (Duitsland) en heeft hij met behulp van een onderzoeksbeurs 2 maanden bij het Instituut voor Virologie gewerkt. Momenteel is hij op zoek naar een baan.

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