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Novel Mass Spectrometry-based Epitope Mapping Procedures of Autoantigens

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Personal Declaration

I declare that this dissertation is the product of my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Reham Elkased

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I. Summary

1. Introduction

1.1 Autoantibodies and Autoantigens

Autoantibodies are antibodies directed towards self antigens and found in autoimmune diseases. It is not certain whether all autoantibodies are pathogenic but many have been confirmed to be involved in the development of autoimmune diseases (Bizzaro, 2007). Independent of their role in pathogenesis they are useful for diagnostic purposes.

It has become clear that in addition to knowing full-length antigens that are related to a certain autoimmune disease, the ability to accurately characterize an epitope structure on an antigen is essential to better understand the pathogenesis of such a disease (Dhungana et al., 2009; Raska et al., 2003) or to improve the diagnostic accuracy of an assay.

Epitopes that are recognized by autoantibodies are structurally diverse, ranging from linear peptide sequences to quaternary structures, and can be modified, hidden, or even mimicked by other structures (Pollard, 2006).

The determination of the amino acid sequence of epitope-containing peptides is of major interest in broad areas of biochemical and biomedical research, as it possesses the potential to define subsets of peptides indicative for marker auto-antibodies of autoimmune diseases (Lorenz et al., 2009; Reichert et al., 2005). Knowledge of individual epitope patterns may eventually be suitable for stratification of patients, possibly leading to guidance for individualized therapy as well as for proteomics and protein characterization studies (Raska et al., 2003).

1.1.1 RA33 autoantigen

The RA33 nuclear antigen is found in rheumatoid arthritis (RA) which is one of the most significant autoimmune diseases affecting 1-2% of the human population. More than 10% of the affected individuals encounter a severe fast progressing course of the disease (Steiner et al., 1996). RA is a chronic inflammatory and highly destructive joint disease characterized by persistent synovial inflammation, cartilage degradation, and bone erosion. The characteristics of RA33 nuclear antigen have been partly but not fully elucidated and work is in progress to unveil its nature. It is of interest that antiRA33 antibodies may appear shortly after the onset of RA and are often detectable before a final diagnosis can be made. Therefore they can provide diagnostic

help in early disease, particularly when rheumatoid factor is negative; in a single case, the presence of the antibody in plasma even preceded disease symptoms by approximately one year (Steiner et al., 1996). So far, it has not been known which regions of RA33 are recognized by autoantibodies (Skriner et al., 1997).

1.1.2 Fibrillarin autoantigen

Fibrillarin; a 34-kDa auto-antigen; is present in all nucleated cell types and derives its name from its localization to both the fibrillar center (FC) and dense fibrillar component (DFC) of the nucleolus (Ochs RL, 1985). Fibrillarin is a target for the spontaneously arising autoantibodies in systemic sclerosis autoimmune disease (Ssc; scleroderma) which is a rare, chronic disorder characterized by degenerative changes and scarring in the skin, joints, and internal organs and by blood vessel abnormalities (Takeuchi et al., 1995).

1.2 Protein structure characterization using mass spectrometry

Mass spectrometric technologies enable profiling of autoantibody responses using biological fluids derived from patients with autoimmune disease (discussed in chapter III). They provide a powerful tool to characterize auto-reactive B-cell responses in diseases including RA, multiple sclerosis, autoimmune diabetes, and systemic lupus erythematosus (SLE). Autoantibody profiling may serve purposes including classification of individual patients and subsets of patients based on their ‘autoantibody fingerprint’, examination of epitope spreading and antibody isotype usage, discovery and characterization of candidate autoantigens, and tailoring antigen-specific therapy.

The advantages of mass spectrometric sample analysis are high sensitivity, high mass accuracy, rapid analysis time and low sample consumption (Hager-Braun and Tomer, 2005). Presently, most mass spectrometers routinely allow analysis of peptides or proteins in the low femtomole level.

1.2.1 Peptide mapping / Peptide mass fingerprint

Protein structures can be characterized by mass spectrometric peptide mapping. This means, proteins are digested with specific proteases and their peptide fragments are characterized and identified by different mass spectrometric techniques, with or without chromatographic peptide separation prior to the mass spectrometric analysis (Bantscheff and Glocker, 2001). Amino acid sequences of known proteins can be recalled from public databases and subjected to software programs like GPMAW (Lighthouse Data, Odense, Denmark). These programs theoretically digest the protein and calculate the

apparent masses of the peptide fragments, generated by cleavage of a distinct protease in the according consensus sequence. By comparison of the apparent masses to the experimental *m/z* values a protein sequence coverage can be clearly identified. A spectrum is called interpreted, when all dominant ion signals are assigned to either belonging to the protein of interest or to major contaminants in the sample.

By contrast, a process called Peptide Mass Fingerprint (PMF) is using databases such as Swiss-Prot and TrEMBL (Mikkat et al., 2004; Sinz et al., 2002) for the identification of unknown protein upon mass spectrometric analysis of its proteolytic fragments. Here, high sequence coverage is not needed and the origin of many ion signals in the spectrum mostly is not identified as long as the identification result is unambiguous.

1.2.2 MS peptide sequencing

In cases of doubt, MS/MS fragmentation gives detailed information about the sequence and/or structure, *i.e.* post-translational modification of the original peptide molecular ion, the precursor ion. Due to fragmentation mainly along the peptide backbone, valuable information about the peptide sequence and its post-translational modifications are readily investigated. Fragment ions arising from MS/MS measurement can be of various types, according to their cleavage along the peptide backbone or from the side chains. If after cleavage the charge stays on the *N*-terminal fragment, the ions are named A, B, or C ions, according to Roepstorff and Fohlmann (Figure 1). If the charge stays on the *C*-terminal fragment the ions are labeled X, Y", or Z' ions (Roepstorff and Fohlman, 1984). Ions of the Y"- and the B series dominate MS/MS spectra when collision induced fragmentation (CID) is applied. Upon electron transfer-induced dissociation (ETD), C and Z' ions are dominating the spectrum (Zubarev, 2003).

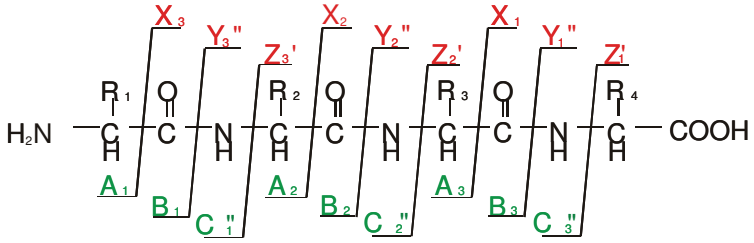

Figure 1: Fragmentation rules for peptides according to Roepstorff and Fohlmann.

Software tools can calculate the apparent masses of the arising fragment ions from given peptide sequences, which can then be compared to the experimental *m/z* values and confirm the supposed peptide sequence. Within the Y" and B fragment ion series the mass differences between adjacent ions corresponds to one amino acid residue. After interpretation of the MS/MS spectrum of an unknown peptide (many times called "de-novo sequencing" (Johnson and Taylor, 2002)) with regard to mass differences, typical for masses of distinct amino acid residues, partial amino acid sequences can be obtained. This information combined with the mass of the peptide can be used for searching in databases (Graves and Haystead, 2002; Koy et al., 2003). In this case the experimentally determined sequence is matched to known sequences, which is by definition not a de-novo sequencing experiment.

1.3 Epitope mapping and epitope prediction

It is important to realize that the epitope nature of a set of amino acids can only be established if an immunoglobulin that is able to bind to it has been found. The immunoglobulin is then called a specific antibody for the antigen that harbours the epitope. By contrast, the antibody nature of an immunoglobulin defined by its paratope becomes apparent even without a complementary known epitope. This sometimes leads to confusion since antigens may possess many different epitopes, but an antibody cannot be specific for the multi-epitopic antigen as a whole but only for one of its epitopes (Van Regenmortel, 1998).

One of the main purposes of identifying epitopes is to replace the epitope regions of intact antigen molecules by linear synthetic peptides that could be used as reagents for detecting anti-protein antibodies in an immunoassay (Leinikki et al., 1993) or as immunogens for raising anti-peptide antibodies able to cross-react with the target protein (Muller, 1999). Further purposes are to develop synthetic peptide vaccines (Van Regenmortel, 2006) or in diagnostics of autoimmune diseases which guide personalized therapies for patients (Lorenz et al., 2009; El-Kased et al., 2010). However, as epitope mapping is difficult to achieve, many different computational-based prediction methods were developed and compared (Pellequer et al., 1991; Ponomarenko and Van Regenmortel, 2009). Unfortunately, none of them, even when used in combination, gave high rates of successful prediction (Odorico and Pellequer, 2003; Blythe and Flower, 2005; Greenbaum et al., 2007). Unsuccessful epitope prediction may partly explain why, after several decades of intensive research efforts, no synthetic peptide vaccine has yet been developed (Hans et al., 2006).

Therefore; robust epitope mapping procedures are of tremendous importance for immunoassay development and production of protective peptide vaccines.

1.3.1 Immuno-analytical methods for epitope mapping

A protein can be cleaved chemically or enzymatically to generate various internal peptides. The number of peptides produced depends on whether the protein is cleaved completely; at many sites; or partially; at limited number of sites. Small numbers of large fragments are produced by limited proteolysis of native proteins. The large fragments are easily separated and purified by either SDS polyacrylamide gel electrophoresis (SDS-PAGE) or high-performance liquid chromatography (HPLC). Proteolytic fragments separated by SDS-PAGE can be electroblotted to an immobilizing membrane and probed with the antibody. The antibody will recognize those fragments containing the epitope. Proteolytic enzymes with different substrate specificity can be used to perform limited digestion of a protein antigen, and antibody binding to the fragments can be analyzed by Western blot (discussed in chapters II and III). When the sequence of the protein is known, the origin of the proteolytic fragments is easily determined after HPLC separation by matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI ToF MS) analysis. A limitation to this procedure is that the denaturated protein and its proteolytic fragments bound to the immobilizing membrane may no longer contain the same assembled and structural antigenic determinants present in the native protein. This technique is not reliable to identify complex assembled epitopes but it is still useful to detect sequential epitopes, consisting of a specific amino acid sequence. Another limitation is the blotting success that depends on the length of the peptide. Shorter peptides are not always bound strong enough for reliable probing for antibody interaction (El-Kased et al., 2009).

1.3.2 Epitope mapping using mass spectrometric analysis

In the case of assembled epitopes, the combination of immuno-precipitation and limited proteolytic digestion of the protein antigen, followed by MALDI ToF MS can be used for determining the antibody-binding region. Proteolytic fragmentation for epitope mapping using MALDI ToF MS analysis can be useful for localization of the antibody-binding region. Another approach to epitope mapping is complete proteolytic digestion of the target protein (discussed in chapter IV).

The choice of complete *vs*. limited proteolysis to localize epitopes depends on several factors. If the unknown region is relatively big, then a limited digest with

subsequent generation of large fragments is preferable. Large protein fragments are also more likely to maintain a more native-like conformation, which may be important for epitope identification. However, many proteins generate multiple products of partial cleavage following limited proteolytic fragmentation. The complex patterns produced by limited proteolysis can be difficult to interpret, making complete proteolytic cleavage advantageous. Complete proteolytic fragmentation is also instrumental when the goal is to localize a specific epitope to the smallest number of amino acid residues possible. Nowadays, the origin of the proteolytic fragments is easily identified using either electrospray ionization time of flight mass spectrometry (ESI ToF) (Rower et al., 2009); or MALDI ToF MS (Mazzoni et al., 2009; Bantscheff and Glocker, 2001).

In order to obtain information on sequential and/or assembled epitopes, various approaches have been developed in conjunction with mass spectrometry. Fine epitope mapping could be achieved by this technique with microgram quantities of antibodies and in a short time compared to conventional mapping methods. Rapid developments in mass spectrometry in combination with the proteolytic digestion of antigen have opened new methods of epitope mapping (Macht et al., 1996; Parker et al., 1996; Suckau et al., 1990). The method is based on the fact that antibodies in general are very resistant to proteolytic digestion.

Epitope excision

In epitope excision the immobilized monoclonal antibody (mAb) is incubated with the whole antigen and the bound antigen is digested with a protease (Figure 2) (Suckau et al., 1990). In general the antibody prevents either proteolysis or chemical modification of sites of the antigen that are situated in the antibody binding pocket. An important feature of this approach is, because non-denaturing conditions are used, that the antigen retains its native conformation so that assembled epitopes can be determined; it also offers the possibility of determining epitopes which contain an enzymatic cleavage site.

Epitope excision and direct identification of a single immune complex by mass spectrometry has shown to be a sensitive and rapid method of high molecular specificity in the analysis of protein antigens. However, so far almost always binary systems were studied, *i.e.* one antigen was complexed with one (monoclonal) antibody solely.

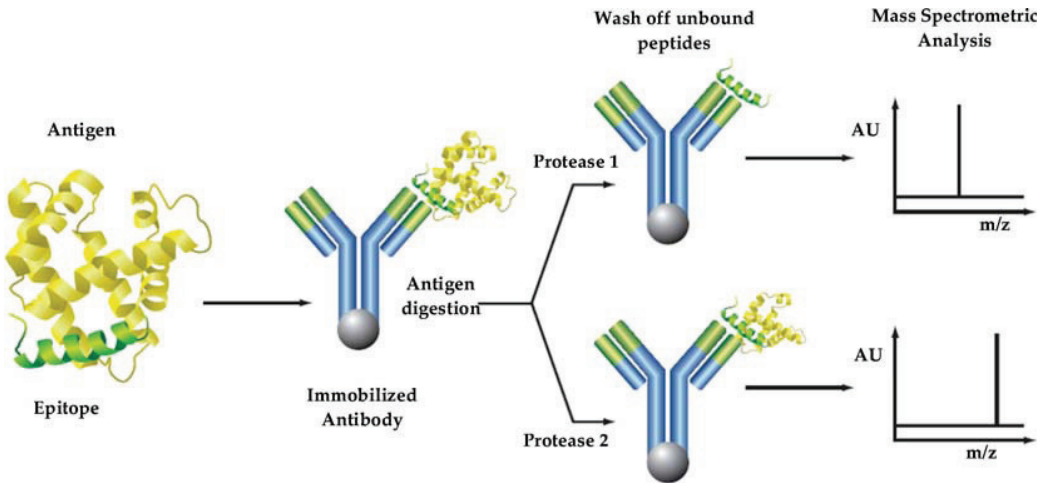

Figure 2: Summary of the epitope excision process. The antigen is bound to the immobilized antibody, followed by digestion with various proteases. Protease 1 and protease 2, which cleave the antigen at different sites, are used for the digestion. Unbound peptides are washed off the column and the affinity-bound peptides are analyzed using mass spectrometry (Dhungana et al., 2009).

Recently, chemical cross-linking of proteins has been combined with mass spectrometric analysis of the created cross-linked products to study the function of a protein and allow the identification of interaction sites with its binding partners (Sinz, 2006; Sinz, 2010). This procedure seems in principle applicable for epitope mapping, when both, the antigen and the antibody, are readily degradable into small peptides and the cross-linked products are analysed. As for most antibodies this is not the case, when using commercially available proteases. Cleavable cross-linkers may be applied in combination with rigid denaturation, but then further work-up steps are needed prior to mass spectrometry, making the procedure difficult to apply outside of expert laboratories.

Epitope extraction

The epitope extraction technique, applied also in chapter IV, is the proteolytic digestion of the free antigen and presentation of the mixture of peptide fragments to the antibody (mostly a mAb), followed by isolation of the antibody-peptide complex (Figure 3). The epitope and non-epitope fractions are analyzed by MALDI-MS, and the epitope sequences are identified directly (Hochleitner et al., 2000; Bílková et al., 2005).

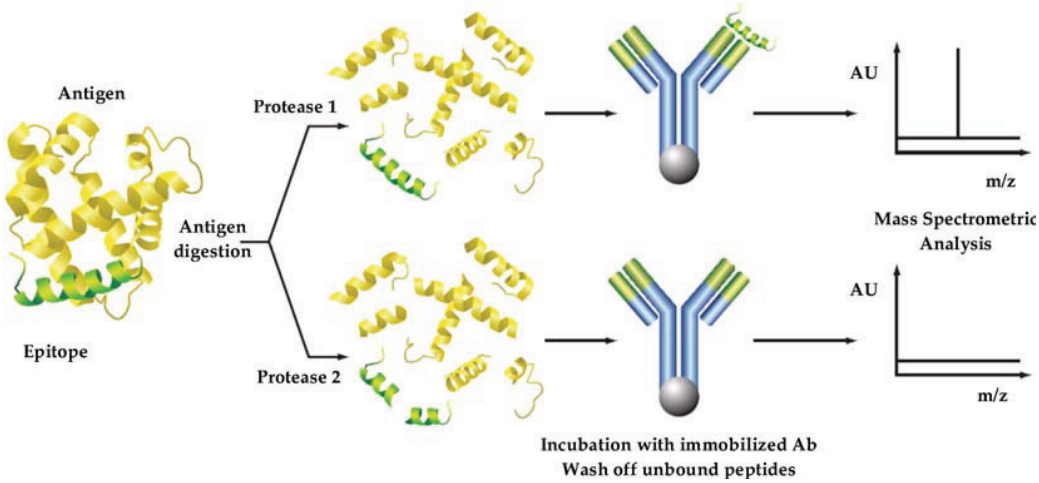

Figure 3: Summary of the epitope extraction process. Protease 1 and protease 2, which cleave the antigen at different sites, are used to digest the antigen. The antigen digest is passed through separate columns containing immobilized antibody. The antigenic determinants of the antigen remain bound to the antibody, while the unbound peptides are washed off from the column. The affinity-bound peptides are analyzed using mass spectrometry (Dhungana et al., 2009).

In both of the above mentioned cases, the peptides containing the epitopes are eluted from immobilized antibody by low pH buffer and analyzed by mass spectrometric peptide fingerprinting in a separate step. The high accuracy and sensitivity of mass spectrometers helps in identifying the peptides by molecular masses and/or peptide sequencing. Clearly, applying immuno-analytical methods coupled with mass spectrometry allows the identification of the epitope region.

2. Research objectives and overview of the thesis

The aim of this study is to establish a robust, direct and easy method to characterize the epitope of an antibody. It will be shown that coupling mass spectrometry-derived findings with peptide chip analysis leads to a precise identification of the epitope peptide of a monoclonal antibody (chapter II) as well as of patients` sera (poly-/oligoclonal antibodies) (chapter III). The applied approach may lead to better stratification of patients, possibly leading to guidance for individualized therapy.

Establishing a novel method for epitope mapping in solution, without immobilization of the antibody, using a manual micro-column size exclusion chromatography coupled with mass spectrometry is discussed in chapter IV as a simplified approach for epitope mapping. The new proposed method yielded in a consistent assignment of the epitope carrying peptide which was previously assigned using immuno-analytical methods combined with mass spectrometric analysis, as applied in chapter II.

3. Practical approach

3.1 Epitope identification using immuno-analytical methods coupled with mass spectrometry

RA33 was subjected to BrCN degradation with subsequent generation of large fragments because the epitope region was unknown. Using few cleavage sites enhances the chances to not disrupt the epitope. The produced fragments were separated on 1D SDS PAGE then the antibody binding to the fragments were analysed by Western blot; where fragments containing the epitope could be assigned to the RA33 protein sequence using mass spectrometry. RA33 degraded with BrCN was also separated with HPLC then the antibody binding to fragments in the separated fractions were analysed by dot blot, where fragments containing the epitope could be assigned to the RA33 protein sequence using mass spectrometry. Both immuno-analytical experiments combined with mass spectrometry lead to the same assignment of the epitope on RA33 protein against which the mAb is directed (section 4.1). The epitope region was confined to the partial amino acid sequence aa 79-163 of the recombinant RA33 using this method.

Using the peptide chip analysis method (section 3.2); synthetic peptides representing the whole RA33 amino acid sequence were synthesized as 15mer peptides overlapping by 9 amino acid residues each. Results obtained from this experiment showed that the epitope was SIDGRVVEP (amino acid range 84 - 92). The results were in agreement with the results obtained from the immuno-analytical methods.

The application of immuno-analytical methods combined with mass spectrometry on detection of epitopes in patients` sera was investigated (chapter III). Poly-/oligoclonal antibodies presented by patients` sera were investigated for the epitope towards which they were directed on RA33. The same procedure as explained above was applied. Again the epitopes assigned by the immuno-analytical methods in combination with mass spectrometry were in good agreement with the peptide chip analysis assignment (section 4.2).

3.2 Peptide chip analysis

High density peptide microarray technologies can be applied in experimental medicine in general and in clinical immunology in particular. Any known sequence can serve as input to synthesize overlapping peptides in the search of disease-associated epitope profiles as preferentially found in sera of patients suffering from autoimmune diseases (Lorenz et al., 2009). The patterns of

bound primary antibodies from the sample are generally visualized using secondary anti-species IgG antibodies conjugated to fluorescence dyes.

High density microarrays consisted of a peptide library spotted in triplicates as 3 identical subarrays. 15mer overlapping peptides were derived from RA33 autoantigen under investigation and for other proteins as well. One subarray carried a total of 9176 peptides from 95 known human autoantigens. These peptide microarrays were incubated with goat anti-species IgG Fab fragments conjugated to AlexaFluor647 (*i.e.* secondary antibody; Invitrogen) and scanned by a Molecular Device GenePix 4000B scanner. Primary data analysis is based on the scanned images and was performed with the GenePix Pro software. Intensities were ranging from 0 to 65536 after subtraction of the background. All data points were recorded in triplicate and data used for further interpretation are mean values.

3.3 Epitope identification using manual size exclusion micro-column chromatography coupled with mass spectrometry

Size exclusion chromatography (SEC) is particularly well suited for separation of proteins from smaller components because it provides easily interpretable qualitative information, *i.e.* the rough molecular weight distribution of a mixture consisting of such components (Sanny, 2002). In addition, SEC is run under non-denaturing conditions which allow the maintenance of the biological activity of the protein (here, the antibody). In SEC, separation is based primarily on size, and as a result, is not noticeably enhanced by the addition of mobile phase modifiers. Resolution in SEC is therefore essentially a sole function of separation efficiency (Lemmo and Jorgenson, 1993). Important developments in SEC have continued in the area of detection systems, mainly light scattering, viscometry, and MALDI ToF mass spectrometry in conjunction with SEC for determining molecular weights and chemical composition of polymeric materials (Barth et al., 1998). Miniaturization of the column used in SEC separations to micro-column dimensions offers various advantages to the technique; such as low sample consumption, low cost per column and reduced maintenance requirements and short duration (Cortes and Pfeiffer, 1993).

The antigens under investigation in this study were subjected to enzymatic proteolysis in order to obtain peptides of masses below 5000 Da. These peptides were subjected to SEC alone or after incubation with mAb in-solution. The epitope-containing peptide, when affinity bound to the antibody and, thus, forming a large complex, eluted early on separation with SEC leaving the

enzymatic cleavage products of the antigenic protein that did not contain the epitope and, hence, were small in size, behind to elute in late fractions. In chapter IV; this novel approach was applied using a manually fabricated micro-column SEC. The approach was first established using two model systems (Fibrillarin and RA33 auto-antigens) containing a His-tag against which anti-His-tag antibodies are directed. Afterwards the approach was applied on the RA33 autoantigen against its mAb (studied in chapter II).

4. Results

4.1 Epitope mapping of RA33 using mAb through coupling of mass spectrometry and immuno-analytical methods

The aim of our study (chapter II) is to characterize the epitope of a monoclonal antiRA33 antibody on recombinant RA33 using mass spectrometric epitope mapping. Recombinant RA33 has been subjected to BrCN cleavage and fragments were separated by SDS PAGE. Subsequent *in-gel* proteolytic digestion and mass spectrometric analysis determined the partial sequences in the protein bands. Western blotting of SDS-PAGE-separated protein fragments revealed immuno-positive, *i.e.* epitope containing bands. BrCN-derived RA33 fragments were also separated by HPLC and immuno-reactivity of peptides was measured by dot-blot analysis with the individual HPLC fractions after partial amino acid sequences were determined. The epitope range was confined to the partial amino acid sequence aa 79-163 of the recombinant RA33. The herewith identified epitope region was compared to data from peptide chip analyses with 15-meric synthetic peptides attached to a glass surface. Results from all three analyses consistently showed that the epitope of the monoclonal anti-RA33 antibody is located in region aa84–92 on recombinant RA33; the epitope sequence is (^{84}SIDGRVVEP^{92}) (El-Kased et al., 2009).

4.2 Epitope mapping of RA33 autoantigen using sera from rheumatoid arthritis patients through coupling of mass spectrometry and immuno-analytical methods

Mass spectrometric epitope mapping in combination with peptide chip analysis (chapter III) showed that autoantibodies from patients who suffered from rheumatoid arthritis (RA) were directed against distinct surface structures on the full-length human autoantigen RA33 as well as against partial sequences. Using the combined mass spectrometric epitope extraction and peptide chip analysis approach, four sequence motifs on RA33 emerged as immuno-positive, showing that epitopes were not randomly distributed on the entire RA33 amino acid sequence. A sequential epitope motif (^{245}GYGGG^{249}) was determined on the *C*-terminal part of RA33 which matched with the Western blot patient screening results using the full-length protein and, thus, was regarded as a disease-associated epitope. Other epitope motifs were found on *N*-terminal partial sequences (^{59}RSRGFGF^{65}, ^{111}KKLFVG^{116}) and again on the *C*-terminal part (^{266}NQQPSNYG^{273}) of RA33. As recognition of these latter three motifs was also recorded by peptide chip analysis using control samples which were negative in the Western blot screening, these latter motifs were regarded as

"cryptic epitopes". Again, mass spectrometry-derived results were in full agreement with the epitope chip data (El-Kased et al., 2010).

4.3 Epitope mapping of proteins using manual size exclusion micro-column chromatography

4.3.1 Development of the in-solution epitope mapping procedure

In order to develop the manual SEC for epitope mapping of antigens two epitopes, His-tags from recombinant proteins, were selected as model epitopes (chapter IV). Recombinant Fibrillarin, a protein derivative comprising amino acid range 76-321 of the full length protein and containing a His-tag at the *N*-terminus, resulted only in partial degradation upon exposure to trypsin. Nevertheless, the generated peptide mixture was found to be suitable for continuing the experimental procedure as nearly full sequence coverage was obtained. SEC experiments of the peptide mixture after incubation with the anti-His antibody showed that a peptide ion signal appeared in the early eluting fractions which was not found in the peptide mixture without incubation with the antibody. Mass spectrometric peptide sequencing analysis of this ion signal revealed its amino acid sequence as being the His-tag-containing *N*-terminal peptide (^{3}GSHHHHHHGSYLGDTIESSTHASGK^{27}), against which the anti-His antibody is directed.

Using the same antibody, the same protease, and the same experimental procedure with a different protein, RA33, again a recombinant protein consisting of 314 amino acids including a His-tag at the *N*-terminus, we were able to identify a peptide ion signal which was present in the early eluting fractions when the tryptic peptide mixture was incubated with the anti-His antibody but was absent in those fractions in the absence of the antibody. Chemically assisted fragmentation-MALDI-MS (CAF-MALDI-MS) and subsequent MS/MS measurements were used to confirm the sequence assignment; where the epitope sequence is (^{1}MSHHHHHHHHSMEREK^{16}) (El-Kased et al., 2011).

4.3.2 Application of in-solution epitope mapping procedure

Thermolysin was the enzyme of choice for RA33 proteolysis as it produced peptides below 4000 Da for all sequence parts throughout the molecule (chapter IV). Comparing the fractions that eluted after SEC experiments of the peptides mixture in the absence of the mAb with those where the antibody was present, one peptide ion signal at *m/z* 1240.01 was observed which shifted from fraction 8 (late eluting in the absence of the antibody) to fraction 3 (early eluting in the presence of it). Together with all control experiments, this shift in elution

suggested that this peptide ion signal was representing the epitope to which the mAb was directed. Mass spectrometric measurements including MS/MS sequencing of this peptide ion signal at *m/z* 1240.01 revealed its amino acid sequence (^{85}IDGRVVEPKRA^{95}). This sequence is the same as previously assigned to being the epitope using the immuno-analytical methods coupled with mass spectrometry and peptide chip analysis (*cf*. chapter II) (El-Kased et al., 2011).

5. Discussion

Several methods have been developed for mapping protein epitopes of mAbs, which involve competition assays (Kuroki, 2009), partial proteolysis (Dhungana et al., 2009), expressing fragments (Böttger and Böttger, 2009), peptide libraries (Reineke, 2009; Bongartz et al., 2009), mass spectrometry (MS) (Dhungana et al., 2009), structure resolution by nuclear magnetic resonance (Rosen and Anglister, 2009) and X-ray crystallography (Sundberg, 2009). Proteolytic fragmentation has been used by us (El-Kased et al., 2009) and others (Pacholczyk and Sweadner 1997) for mapping protein epitopes of mAbs.

In our study an epitope extraction approach was applied for the identification of the epitope on the RA33 autoantigen against which a commercially available mAb is directed. All three sets of independent experimental data (see above) yielded in consistent assignments of epitope carrying peptides, showing that epitopes which are determined by peptide chip analysis are matching with the epitope regions that are found on the full-length protein as well.

Our approach that was making use of the epitope extraction procedure with sera from patients that contained poly-/oligoclonal (auto)antibodies in conjunction with analyzing immuno-response of patient sera with peptide chips and with the combined mass spectrometric epitope extraction procedure identified four major sequence motifs on RA33 emerging as immuno-positive, and hence, showing that epitopes were not randomly distributed on the entire RA33 amino acid sequence.

Recently, it was suggested that antiRA33 autoantibodies in patients may be directed against different epitopes and that the recognition pattern was associated with the disease (Schett et al., 2009). Truncated recombinant RA33 protein derivatives and RA33-derived synthetic peptides were tested with patient sera for immuno-positive reactions and lead to the conclusion that RA patients´ autoantibodies against RA33 recognized an assembled, *i.e.* a conformational epitope that was placed on domain II (Skriner et al., 1997). The presence of (auto)antibodies against sequential epitopes on RA33 in RA patient sera has as yet not been addressed. By contrast, it was suggested that antiRA33 autoantibodies from SLE patients were predominantly directed against a sequential, *i.e.* a linear epitope of RA33 comprising aa155-175 (Schett et al., 2009). Hence, characterizing the epitope recognition specificities of autoantibodies in addition to the determination of the presence of autoantibodies against full-length antigens in autoimmune patients´ sera is regarded to be extremely helpful to improve the diagnostic and even prognostic

value of a screening method which may guide individual therapy and on which therapy-related decisions may be based on.

In our third approach we wished to develop a simplified method for assigning an epitope which would have several characteristics; the reaction would occur in solution which would avoid certain artefacts and be similar to the reaction of antibody and soluble antigens in many *in vivo* situations. From a practical standpoint the method should be easily applied with the instrumentation usually found in the average protein-analytical laboratory.

As most antibody-epitope complexes are quite stable, size exclusion chromatography was thought to provide a rapid means for epitope assignment. The development of a method using manual size exclusion micro-column chromatography to assign the epitope was initiated in this study with two model systems composed of recombinant His-Tag-containing antigens in combination with a monoclonal anti-His-tag antibody. Later the method was extended to identify an epitope within the recombinant RA33 autoantigen against which a monoclonal antiRA33 antibody was directed.

It should be noted that a purified antigen protein is not required for this procedure, because only peptides containing the antigenic determinant will bind to the antibody in the initial step. The epitope-containing fragment, affinity bound to the antibody and, thus, forming a complex which elutes early on SEC, will be separated efficiently from the other enzymatic cleavage products of the antigen protein not containing the epitope. The latter remain small in size and elute late. Dissociation of the antigen-antibody complex prior to mass spectrometric analysis is unnecessary because the affinity-bound epitope peptides are released during the MALDI matrix crystallization process.

6. References

Bantscheff, M., and Glocker, M.O. (2001). MALDI-Massenspektrometrie in der Proteomforschung Biospektrum 7, 250-252.

Barth, H.G., Boyes, B.E., and Jackson, C. (1998). Size Exclusion Chromatography and Related Separation Techniques. Anal Chem 70, 251-278.

Bílková, Z., Stefanescu, R., Cecal, R., Korecká, R., Ouzká, Š., Ježová, J., Viovya, J.-L., and Przybylski, M. (2005). Epitope extraction technique using a proteolytic magnetic reactor combined with Fourier-transform ion cyclotron resonance mass spectrometry as a tool for the screening of potential vaccine lead peptides. Eur J Mass Spectrom 11, 489-495.

Bizzaro, N., Tozzoli, R., and Shoenfeld, Y. (2007). Are we at a stage to predict autoimmune rheumatic diseases? . Arthritis Rheum 56, 1736-1744.

Blythe, M.J., and Flower, D.R. (2005). Benchmarking B cell epitope prediction: Underperformance of existing methods. Protein Sci 14, 246-248.

Bongartz, J., Bruni, N., and Or-Guil, M. (2009). Epitope Mapping Using Randomly Generated Peptide Libraries. In Epitope Mapping Protocols M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 237 - 246.

Böttger, V., and Böttger, A. (2009). Epitope Mapping Using Phage Display Peptide Libraries. . In Epitope Mapping protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 181 - 202.

Cortes, H.J., and Pfeiffer, C.D. (1993). Microcolumn size exclusion chromatography with polymeric stationary phases. Anal Chem 65, 1476-1480.

Dhungana, S., Williams, J.G., Fessler, M.B., and Tomer, K.B. (2009). Epitope Mapping by Proteolysis of Antigen–Antibody Complexes. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 87-101.

El-Kased, R.F., Koy, C., Lorenz, P., Montgomery, H., Tanaka, K., Thiesen, H.-J., and Glocker, M.O. (2011). A Novel Mass Spectrometric Epitope Mapping Approach Without Immobilization of the Antibody. J Proteomics Bioinform 4, 001-009.

El-Kased, R.F., Koy, C., Lorenz, P., Drynda, S., Guthke, R., Qian, Z., Koczan, D., Li, Y., Kekow, J., Thiesen, H.-J., and Glocker, M.O. (2010). Mass Spectrometric and Peptide Chip Epitope Analysis on the RA33 Autoantigen with Sera from Rheumatoid Arthritis Patients. Eur J Mass Spectrom 16, 443 - 451.

El-Kased, R.F., Koy, C., Deierling, T., Lorenz, P., Qian, Z., Li, Y., Thiesen, H.-J., and Glocker, M.O. (2009). Mass Spectrometric and Peptide Chip Epitope Mapping of Rheumatoid Arthritis Autoantigen RA33. Eur J Mass Spectrom 15, 747 - 759.

Graves, P.R., and Haystead, T.A.J. (2002). Molecular Biologist's Guide to Proteomics. Microbiol Mol Biol Rev 66, 39-63.

Greenbaum, J.A., Andersen, P.H., Blythe, M., Bui, H.H., Cachau, R.E., Crowe, J., Davies, M., Kolaskar, A.S., Lund, O., Morrison, S., et al. (2007). Towards a consensus on datasets and evaluation metrics for developing B-cell epitope prediction tools. J Mol Recognit 20, 75-82.

Hager-Braun, C., and Tomer, K.B. (2005). Determination of protein-derived epitopes by mass

spectrometry. Expert Rev Proteomics 2, 745-756.

Hans , D., Young , P.R., and Fairlie , D.P. (2006). Current status of short synthetic peptides as vaccines. Med Chem 2, 627 - 646.

Hochleitner, E.O., Gorny, M.K., Zolla-Pazner, S., and Tomer, K.B. (2000). Mass Spectrometric Characterization of a Discontinuous Epitope of the HIV Envelope Protein HIV-gp120 Recognized by the Human Monoclonal Antibody 1331A. J Immunol 164, 4156-4161.

Johnson, R.S. and Taylor, J.A. (2002). Searching sequence databases via de novo peptide sequencing by tandem mass spectrometry. Mol Biotechnol 22, 301-315.

Koy, C., Mikkat, S., Raptakis, E., Sutton, C., Resch, M., Tanaka, K., and Glocker, M.O. (2003). Matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight mass spectrometry sequencing resolves structures of unidentified peptides obtained by in-gel tryptic digestion of haptoglobin derivatives from human plasma proteomes. Proteomics 3, 851-858.

Kuroki, M. (2009). Solid-Phase Mutual Inhibition Assay with Labeled Antigen. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 59 - 66.

Leinikki , P., Lehtinen , M., Hyöty , H., Parkkonen , P., Kantanen , M.L., and Hakulinen , J. (1993). Synthetic peptides as diagnostic tools in virology. Adv Virus Res 42, 149 - 186.

Lemmo, A.V., and Jorgenson, J.W. (1993). Two-dimensional protein separation by microcolumn size-exclusion chromatography-capillary zone electrophoresis. J Chromatogr A 633, 213-220.

Lorenz, P., Kreutzer, M., Zerweck, J., Schutkowski, M., and Thiesen, H.-J. (2009). Probing the Epitope Signatures of IgG Antibodies in Human Serum From Patients With Autoimmune Disease. In Methods in Molecular Biology Epitope Mapping Protocols, U. Reineke, and M. Schutkowski, eds. (Humana Press), pp. 247-258.

Macht, M., Fiedler, W., Kürzinger, K., and Przybylski, M. (1996). Mass spectrometric mapping of protein epitope structures of myocardial infarct markers myoglobin and troponin T. Biochemistry 35, 15633-15639.

Mazzoni, M.R., Porchia, F., and Hamm, H.E. (2009). Proteolytic Fragmentation for Epitope Mapping. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 77 - 86.

Mikkat, S., Koy, C., Ulbrich, M., Ringel, B., and Glocker, M.O. (2004). Mass spectrometric protein structure characterization reveals cause of migration differences of haptoglobin α chains in two-dimensional gel electrophoresis. Proteomics 4, 3921-3932.

Muller , S. (1999). Use of antipeptide antibodies in molecular and cellular biology. In Synthetic Peptides as Antigens. M. H. V. Van Regenmortel, and S. Muller, eds. (Elsevier), pp. 215 - 235.

Ochs RL , L.M., Spohn WH , Busch H (1985). Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell 54, 123-133.

Odorico, M., and Pellequer, J.L. (2003). BEPITOPE: predicting the location of continuous epitopes and patterns in proteins. J Mol Recognit 16, 20-22.

Pacholczyk , T., and Sweadner , K.J. (1997). Epitope and mimotope for an antibody to the Na, K-ATPase. Protein Sci 6, 1537 - 1548.

Parker, C., Papac, D., Trojak, S., and Tomer, K. (1996). Epitope mapping by mass spectrometry:

determination of an epitope on HIV-1 IIIB p26 recognized by a monoclonal antibody. J Immunol 157, 198-206.

Pellequer, J.L., Westhof, E., Van Regenmortel, M.H.V., and John, J.L. (1991). [8] Predicting location of continuous epitopes in proteins from their primary structures. In Methods in Enzymology (Academic Press), pp. 176-201.

Pollard, K.M. (2006). Perspectives. In Autoantibodies and Autoimmunity: Molecular Mechanisms in Health and Disease, K.M. Pollard, ed. (Wiley-VCH Verlag GmbH & Co. KGaA), pp. 591-598.

Ponomarenko, J.V., and Van Regenmortel, M.H.V. (2009). B cell epitope prediction. In Structural Bioinformatics, P.E. Bourne, and G. J., eds. (Hoboken, NJ, John Wiley), pp. 849-879.

Raska, C.S., Parker, C.E., Sunnarborg, S.W., Pope, R.M., Lee, D.C., Glish, G.L., and Borchers, C.H. (2003). Rapid and sensitive identification of epitope-containing peptides by direct matrix-assisted laser desorption/ionization tandem mass spectrometry of peptides affinity-bound to antibody beads. J Am Soc Mass Spectrom 14, 1076-1085.

Reichert, J.M., Rosensweig, C.J., Faden, L.B., and Dewitz, M.C. (2005). Monoclonal antibody successes in the clinic. Nat Biotech 23, 1073-1078.

Reineke, U. (2009). Antibody Epitope Mapping Using De Novo Generated Synthetic Peptide Libraries. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 203-211.

Roepstorff, P., and Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom 11, 601.

Rower, C., Vissers J.P.C., Koy,C., Kipping,M., Hecker,M., Reimer,T., Gerber,B., Thiesen,H-J., and Glocker, M.O. (2009). Towards a proteome signature for invasive ductal breast carcinoma derived from label-free nanoscale LC-MS protein expression profiling of tumorous and glandular tissue. Anal Bioanal Chem 395, 2443-2456.

Rosen, O., and Anglister, J. (2009). Epitope Mapping of Antibody-Antigen Complexes by Nuclear Magnetic Resonance Spectroscopy. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 37 - 58.

Sanny, C.G. (2002). Review: Antibody-antigen binding study using size-exclusion liquid chromatography. J Chrom B 768, 75-80.

Schett, G., Dumortier, H., Hoefler, E., Muller, S., and Steiner, G. (2009). B cell epitopes of the heterogeneous nuclear ribonucleoprotein A2: identification of a new specific antibody marker for active lupus disease. Ann Rheum Dis 68, 729-735.

Sinz, A. (2010). Investigation of Protein-Protein Interactions in Living Cells by Chemical Crosslinking and Mass Spectrometry. Anal Bioanal Chem 397, 3433-3440.

Sinz, A. (2006). Chemical Crosslinking and Mass Spectrometry to Map Three-Dimensional Protein Structures and Protein-Protein Interactions. Mass Spectrom Rev 25, 663-682.

Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B., Drynda, S., Kekow, J., Thiesen, H.J., and Glocker, M.O. (2002). Mass spectrometric proteome analyses of synovial fluids and plasmas from patients suffering from rheumatoid arthritis and comparison to reactive arthritis or osteoarthritis. Electrophoresis 23, 3445-3456.

Skriner, K., Sommergruber, W.H., Tremmel, V., Fischer, I., Barta, A., Smolen, J.S., and Steiner,

G. (1997). Anti-A2/RA33 Autoantibodies Are Directed to the RNA Binding Region of the A2 Protein of the Heterogeneous Nuclear Ribonucleoprotein Complex. J Clin Invest 100, 127-135.

Steiner, G., Skriner, K., Hassfeld, W., and Smolen, J.S. (1996). Clinical and immunological aspects of autoantibodies to RA33/hnRNP-A/B proteins-a link between RA, SLE and MCTD. Mol Biol Rep 23, 167-171.

Suckau, D., Kohl, J., Karwath, G., Schneider, K., Casaretto, M., Bitter-Suermann, D., and Przybylski, M. (1990). Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping. Proc Natl Acad Sci USA 87, 9848-9852.

Sundberg, E.J. (2009). Structural Basis of Antibody-Antigen Interactions. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 23 - 36.

Takeuchi, K., Turley, S., Tan, E., and Pollard, K. (1995). Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity. J Immunol 154, 961-971.

Van Regenmortel, M.H.V. (1998). Mimotopes ,Continuous paratopes and hydropathic complementarity: Novel approximations in the description of immunochemical specificity. J Dispers Sci Technol 19, 1199 - 1219.

Van Regenmortel, M.H.V. (2006). Immunoinformatics may lead to a reappraisal of the nature of B cell epitopes and of the feasibility of synthetic peptide vaccines. J Mol Recognit 19, 183-187.

Zubarev, R.A. (2003). Reactions of polypeptide ions with electrons in the gas phase. Mass Spectrom Rev 22, 57-77.

II. Mass Spectrometric and Peptide Chip Epitope Mapping of Rheumatoid Arthritis Autoantigen RA33

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1. Abstract

The protein termed RA33 was determined to be one major autoantigen in Rheumatoid Arthritis patients and antiRA33 autoantibodies were found to appear shortly after onset of RA. They often are detectable before a final diagnosis can be made in the clinic. The aim of our study is to characterize the epitope of a monoclonal antiRA33 antibody on recombinant RA33 using mass spectrometric epitope mapping. Recombinant RA33 has been subjected to BrCN cleavage and fragments were separated by SDS PAGE. Subsequent in-gel proteolytic digestion and mass spectrometric analysis determined the partial sequences in the protein bands. Western blotting of SDS PAGE-separated protein fragments revealed immuno-positive, *i.e.* epitope containing bands. BrCN-derived RA33 fragments were also separated by HPLC and immuno-reactivity of peptides was measured by dot-blot analysis with the individual HPLC fractions after partial amino acid sequences were determined. The herewith identified epitope region was compared to data from peptide chip analysis with 15-meric synthetic peptides attached to a glass surface. Results from all three analyses consistently showed that the epitope of the monoclonal antiRA33 antibody is located in the region aa79–84 on recombinant RA33; the epitope sequence is MAARPHSIDGRVVEP. Sequence comparisons of the 15 best scoring peptides from the peptide chip analysis revealed that the epitope can be separated into two adjacent binding parts. The *N*-terminal binding parts comprise the amino acid residues “DGR” resembling the general physicochemical properties “acidic/polar–small–basic”. The *C*-terminal binding spots contain the amino acid residues “VVE” with the motif “hydrophobic–gap–acidic”. The matching epitope region that emerged from our analysis on both, the full-length protein and the 15-meric surface bound peptides suggests that peptide chips are indeed suitable tools for screening patterns of autoantibodies in patients suffering from autoimmune diseases.

Keywords

autoimmune diseases; RA33 autoantigen; monoclonal antibody; mass spectrometry; epitope mapping; peptide chip analysis

Abbreviations

aa: amino acid
DHB: 2,5-dihydroxy benzoic acid;
CHCA: α-cyano-4-hydroxy cinnamic acid
RA33: rheumatoid arthritis autoantigen
TBS: TRIS buffered saline
ACN: acetonitrile

2. Introduction

Rheumatoid arthritis (RA) is one of the most significant chronic diseases affecting ca. 1 % of the human population. It is characterized by chronic inflammation of the synovial joints which leads to progressive joint erosions and eventually to disability[1-3]. Over the past years, several autoantibodies have been described in patients with RA (*e.g.* antiperinuclear factor antibodies, antikeratin antibodies, antiRA33 antibodies) but not all have been successfully incorporated into routine clinical practice[4]. From them, RA33 was determined to be one major autoantigen in RA patients, but patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) have been found to also possess autoantibodies against RA33 to some extent. However, these latter diseases differ in their reactivities to other spliceosomal proteins. Therefore, it has been postulated that antiRA33 autoantibodies are not only valuable diagnostic markers but may also allow additional insights into the pathogenesis of rheumatic autoimmune diseases[5].

RA33 carries its name according to its migration behaviour in SDS PAGE and has been found to be identical to the protein named hnRNP A2/B1 (RefSeq Accessions NP_002128 and NP_112533; Uniprot Accession number P22626). HnRNP proteins are associated with pre-mRNAs in the nucleus in complex 4 which forms part of the spliceosome[6]. They are involved in pre-mRNA processing and other aspects of mRNA metabolism and transport.

It is of particular interest that antiRA33 autoantibodies may appear shortly after the onset of RA and are often detectable before a final diagnosis can be made in the clinic. It has been reported that the presence of antiRA33 antibodies may predict disease symptoms by one year[5]. Therefore they can provide diagnostic help early in disease, particularly when Rheumatoid Factor (RF) is negative in patient samples. In conjunction with anti-CCP antibody level determination, measuring antiRA33 autoantibody levels in RA patients and in other autoimmune patients has become important in particular for diagnosing RA in the absence of RF and for estimating severity of the disease[6,7]. Predicting severity of disease development is directing ongoing and future therapy.

It has become clear that in addition to knowing full-length antigens that are related to a certain autoimmune disease, the ability to accurately characterize an epitope structure on an antigen is essential to better understand the pathogenesis of such a disease[8,9]. The determination of the amino acid sequence of epitope-containing peptides is of major interest in broad areas of biochemical and biomedical research, as it possesses the potential to define

subsets of peptides indicative for marker autoantibodies of autoimmune diseases[10]. Knowledge of individual epitope patterns may eventually be suitable for stratification of patients, possibly leading to guidance for individualized RA therapy.

In our study an epitope extraction approach was applied for the identification of the epitope on RA33 against which a commercially available monoclonal antibody is directed. RA33 has been degraded by BrCN and fragments were isolated by SDS PAGE. The partial sequences in the protein bands were determined by subsequent *in-gel* proteolytic digestion and mass spectrometric analysis. Combination with Western blotting revealed these fragments which were immuno-positive, *i.e.* contained the epitope. In addition, we separated BrCN-derived RA33 fragments by HPLC and analyzed the immuno-reactivity of peptides by dot-blot analysis whose partial amino acid sequences first were determined by mass spectrometry in the individual HPLC fractions. At last, the antibody-epitope peptide interaction was characterized using surface-bound 15-mer peptides on chips to which the antibody was administered. All three sets of independent experimental data yielded in consistent assignments of epitope carrying peptides, showing that epitopes determined by peptide chip analysis are matching with the epitope regions that are found on the full-length protein as well.

3. Materials and Methods

3.1 HPLC separation of RA33 and BrCN fragments

Recombinant RA33 protein was purchased from Euroimmun AG, Luebeck, Germany. This autoantigen was dissolved in 50 mM sodium phosphate, pH 7.4; 8 M urea; and 1000 mM sodium chloride with a final concentration of 2.42 mg/ml. HPLC was performed to remove salt extents from the recombinant RA33 protein. The HP 1100 chromatography system (Agilent, Boeblingen, Germany) was used, equipped with an UltraSep ESD 300 PROT RP4S 125 x 2 mm end-capped column. 100 µl of sample were loaded and flow rate was set to 0.5 ml/min. Solvent A consisted of 0.1 % TFA in water and solvent B was 0.1 % TFA in acetonitrile. The following gradient was applied: from 0 to 20 % B in 55 min, then to 80 % B in 5 min, 20 % B was reached in 10 min. The protein-containing fraction was collected and lyophilized in a speed-vac concentrator (Savant Instruments, Holbrook, NY, USA).

The BrCN-derived fragments of RA33 were separated using the HPLC system

as described above with a solvent gradient from 0 to 10 % B in 10 min, then to 80 % B in 70 min, 80 % B was kept for 10 min, then 10 % B was reached in 10 min. An UltraSep ES PEPTID 125 x 2 mm column was used and flow rate was set to 0.5 ml/min. The separated and purified fractions were collected in Eppendorf LowBind tubes and lyophilized.

3.2 BrCN degradation of RA33

For BrCN degradation, HPLC purified and lyophilized RA33 (ca. 100 µg) was re-dissolved in 30 µl of 80 % ACN/0.1 % TFA solution. 10 µl of 5 M BrCN solution was added and incubated for 20 h at 25 °C in the dark[11]. The mixture of BrCN-derived fragments was lyophilized. Samples containing BrCN-derived fragments of RA33 were subjected to mass spectrometry, to HPLC and to SDS PAGE, respectively.

3.3 SDS PAGE analysis of BrCN fragments of RA33 and in-gel tryptic digest

The lyophilized BrCN-derived fragments of RA33 were resuspended in 30 µl of an aqueous 80 % ACN/0,1 % TFA solution, then took 5 µl and mixed with 5 µl of a 2.5fold sample buffer containing 156 mM TRIS, 5 % SDS, 25 % glycerol, and 12,5 % 2-mercaptoethanol. This sample solution (10 µl) was loaded onto a 4-20 % TRIS glycine gel, 1.0 mm x 15 well (Invitrogen, Karlsruhe, Germany). The Broad Range Marker (New England BioLabs, Frankfurt/Main, Germany) as well as the Ultralow Range Marker (Sigma, Munich, Germany) were used for determining apparent molecular masses of the protein bands. Gels were run in MOPS buffer (0.025 M MOPS, 0.025 M TRIS, 3.465 mM SDS, 1.025 mM EDTA) and using the Xcell Surelock^{TM} MiniCell electrophoresis chamber (Invitrogen, Karlsruhe, Germany) at a constant voltage of 200 V for 30 min. Gels were fixed with a 50 % ethanol/10 % acetic acid/water solution and stained with colloidal Coomassie Brillant Blue G-250[12]. Protein bands were manually excised and gel plugs were subjected to *in-gel* digestion with trypsin (Promega, Madison, WI, USA) and Asp-N (Roche Diagnostics, Mannheim, Germany), respectively[13]. For every gel plug, 5 µl of the enzyme solution dissolved in 1 ml of a TRIS/HCl solution (3 mM, pH 8.5) was added per well and incubated for 5 h at 37 °C. Peptide mixtures were extracted with 5 µl of an aqueous solution consisting of 50 % ACN, 0.3 % TFA, and 5 mM *N*-octylglucopyranoside.

3.4 Sample preparation for mass spectrometry

Lyophylized intact RA33 from HPLC purification (*ca.* 3 nmol) was re-dissolved in 80 % ACN/0.1 % TFA (30 µl), vigorously shaken for at least 10 min and

centrifuged for 5 min at 13,000 rpm, 25 °C. For molecular mass determination and for ISD measurements, 0.5 µl of protein solutions were prepared onto a stainless steel target applying ferulic acid (saturated solution in 1 ml of an aqueous ACN/0.1 % TFA 33/67 v/v) and DHB (12.5 mg/ml in 50 % ACN/0.1 % TFA), respectively, as matrices. 1 µl of the ferulic acid solution was pipetted onto the target, left there for some seconds and removed. 0.5 µl of the sample solution were spotted on the target and 0.5 µl of ferulic acid solution were added and mixed with the sample solution directly on the target. The mixture was then allowed to dry on air.

For ISD measurements, 0.5 µl of the RA33 solution were pipetted onto the target. 1.0 µl of DHB solution (12.5 mg DHB dissolved in 1 ml 50 %ACN/0.1 % TFA) was added, mixed directly on the target and air-dried. For peptide mass fingerprinting, sample preparations were carried out on an AnchorChip^{TM} 600/384 target plate (Bruker Daltonik, Bremen, Germany) applying CHCA as matrix[14].

3.5 MALDI ToF mass spectrometry

MALDI ToF MS measurements were performed using a Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with the SCOUT source and operated in positive ion linear mode. MALDI was carried out utilizing a nitrogen pulsed laser (337 nm, 3-5 ns pulse width). A total of 400 laser shots were accumulated producing sum spectra ranging from *m/z* 4000-80,000. Spectra were externally calibrated using the commercially available Protein Calibration Standards I and II (Bruker Daltonik, Bremen, Germany). ISD was performed using the following parameters: IS1: 20.00 kV, IS2: 16.35 kV, Lens: 9.8 kV, Reflector: 23 kV, mass range: *m/z* 1000-10,000. ISD spectra were calibrated using ISD signals from myoglobin (Sigma, Munich, Germany)[15,16].

The *in-gel* digested tryptic peptides and the BrCN mixtures were analyzed in positive ion reflector mode. Spectra were externally calibrated with a commercially available Peptide Calibration Standard (Bruker Daltonik, Bremen, Germany) and internally re-calibrated using peptide ion signals of known masses of BrCN fragments or selected peptide ion signals from trypsin auto-proteolysis with the following masses: ($[M+H]^+$ 842.51, $[M+H]^+$ 1045.56, $[M+H]^+$ 2211.11, $[M+H]^+$ 2807.39). Spectra were analyzed using both the FlexAnalysis 2.4 and BioTools 3.0 programs (Bruker Daltonik, Bremen, Germany).

3.6 MALDI QIT ToF MS/MS sequencing

MS/MS spectra were acquired with an Axima MALDI QIT ToF mass

spectrometer (Shimadzu Biotech, Manchester, UK) in positive ion mode utilizing a nitrogen pulsed laser (337 nm, 3-5 ns peak-width full width half maximum, FWHM) and employing a three-dimensional quadrupole ion trap supplied by helium (pulsed flow gas) for collisional cooling and argon (collisional gas) to impose collisionally induced dissociation (CID)[17,18]. For MS/MS measurements 0.5 µl of the DHB solution (0.005 g dissolved in 1 ml of an aqueous ACN/0.1 % TFA (33/67 v/v) solution were spotted onto an AnchorChip™ 400/384 target plate (Bruker Daltonik, Bremen, Germany). 0.5 µl of the digested peptide mixtures were added, mixed directly on the target and air-dried. Spectra were externally calibrated with a manually mixed peptide standard consisting of bradykinin (1-7) $[M+H]^+$ 757.39, angiotensin II $[M+H]^+$ 1046.53, angiotensin I $[M+H]^+$ 1296.68, bombesin $[M+H]^+$ 1619.81, *N*-acetyl renin substrate $[M+H]^+$1800.93, ACTH (1-17) $[M+H]^+$ 2093.08, ACTH (18-39) $[M+H]^+$ 2465.19, somatostatin $[M+H]^+$ 3147.46, insulin (oxidized beta chain) $[M+H]^+$ 3494.64 as well as internally re-calibrated using known ion signals of the MS/MS spectrum. Further processing and analysis of the MS/MS spectra were performed with the Launchpad™ software, version 2.7.1 (Shimadzu Biotech, Manchester, UK).

3.7 Immunoanalytical Analysis with antiRA33 mAb

For Western blot analysis 1D SDS PAGE was performed as described above with BrCN-derived fragments. Separated protein fragments were blotted onto a PVDF membrane (Immobilon, Millipore, Schwalbach, Germany) by semi-dry blotting for 60 min with a current of 1.2 mA/cm^2. The membrane was cut into strips. Strips were blocked with 2 ml blocking buffer (TBS 5 % non-fat dry milk powder, 1 % BSA 0.02 % thimerosal) for 2 h. Then the monoclonal antibody (mouse antihnRNP-A2/B1, suspended in 0.01 M PBS, pH 7.4, 15 mM sodium azide, antibody concentration: ca. 1.5 mg/ml, Sigma, Munich, Germany) was diluted 1:1900 with blocking buffer. This antibody solution (2 ml per strip) was added to the strips after blocking and incubated over night at 4 °C. Washing (three times with 2 ml per strip with a buffer consisting of TBS, 0.05 % TWEEN 20 (v/v), 0.1 % BSA (w/v), 0.02 % thimerosal (w/v)) was followed by secondary antibody reaction (biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG+IgM (H + L), freeze-dried powder rehydrated in 1.5 ml of water (antibody concentration: 1.1 mg/ml in 0.01 M sodium phosphate, 0.25 M sodium chloride, pH 7.6, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA/Dianova). For this purpose the secondary antibody solution was diluted 1:20,000 with blocking buffer. 2 ml per strip were added and incubated for 1 h at room temperature. Another washing step and reaction with 2 ml per strip of a 1:10,000 dilution with blocking buffer of a Peroxidase-conjugated Streptavidin

solution (freeze-dried powder rehydrated in 1 ml water (concentration: 1 mg/ml in 0.01 M sodium phosphate, 0.25 M sodium chloride, pH 7.6, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA/Dianova) was carried out for 1 h at room temperature. Detection was performed using the SuperSignal West Pico Chemiluminiscent substrate (Pierce/ThermoFisher Scientific, Bonn, Germany).

Lyophilized HPLC fractions of BrCN-derived fragments of RA33 were re-dissolved in 310 µl of a buffer containing 4 M urea, 1 % CHAPS, 8 mM DTT, and 0.25 % IPG buffer each and 100 µl per well were added onto a PVDF membrane using the MINIFOLD dot blotting device (Schleicher & Schuell/ Whatman, Dassel, Germany) together with intact RA33 (1 µl of the stock solution as delivered with a final concentration of 2.42 mg/ml, was mixed with 309 µl of the same buffer) as a positive control and a blank (water) as a negative control. After the dot blot procedure, the membrane was taken out of the device and the blocking as well as the antibody incubation procedure was performed as described above.

3.8 Peptide Chip Analysis with antiRA33 mAb

Replitope™ high density microarrays were custom-made at JPT Peptide Technologies GmbH, Berlin, Germany. Their layout consisted of the whole peptide library spotted in triplicate as 3 identical subarrays. One subarray carried a total of 9176 different 15mer peptides overlapping by 9 amino acid residues that reflected the amino acid sequences of 95 known human autoantigens. Peptide sequences were included derived from the RA33 autoantigen isoforms (heterogeneous nuclear ribonucleoprotein hnRNPA2/B1 RefSeq Accessions NP_002128 and NP_112533 (P22626)) as well as from hnRNPA1 (RefSeq NP_002127, NP_112420 (P09651)) and hnRNPAB (RefSeq NP_112556, NP_004490 (Q99729)) proteins. Details on peptide synthesis, chip layout, staining, processing and evaluation have been described previously[10]. Here, the chips were used to characterize the epitope binding profiles of a monoclonal antibody against RA33 (mouse Ig2a antihnRNPA2/B1, Sigma R4653). Incubations with antibody solutions were done in a chamber formed by a 2-slide sandwich in duplicate. The incubation volume was 500 µl for each step. Primary and secondary antibody samples were dissolved in a buffer consisting of TBS (50 mM TRIS/HCl pH 7.4, 150 mM sodium chloride), 0.05 % Tween 20, and 0.1 % BSA and incubated for 4 h at room temperature. The final concentration of the antiRA33 antibody was 3.8 µg/ml. The secondary antibody was a polyclonal Fab-fragment goat IgG preparation directed against human

IgG and conjugated with AlexaFluor647 fluorescent dye (Invitrogen Molecular Probes Z25408) and was used at 0.8 µg/ml concentration. After each antibody incubation step the chips were washed with buffer; 3 times for 5 min, each. Fluorescence readings were acquired at 10 µm resolution with a scanner that was equipped with a 635 nm laser and a 532 nm laser for excitation (Axon Instruments GenePix 4000B, Molecular Devices; Ismaning, Germany). TIFF format images with a resolution of 2540 dpi at 16 bit depth were analyzed with GenePix Pro software version 4.0 (Molecular Devices) using the chip layout file (GenePix Array List GAL file) with spot-specific information. The median fluorescence and the respective background at 635 nm excitation of each feature were used to generate a list of signals specific for the peptides on the chip. An image depth of 16 bit means that each pixel can cover signal intensities representing a grey value distribution from 0 up to 65536.

4. Results

4.1 Structural characterization of recombinant RA33

The recombinant RA33 protein under investigation is consistent with the human hnRNP A2/B1 protein (Acc. No. P22626) except for the *N*-terminal His-Tag (aa1-11) and a sequence gap at position aa 250 | aa251 (aa 240-279 in hnRNP A2/B1), resulting in a short RA33 form like the one that is known in mouse (hnRNP A2/B1 isoform 3; Acc. No. O88569). The sequence from the mouse protein hnRNP A2/B1 isoform 3 matches well with the sequence of the human protein except for a single amino acid exchange; N258S. The recombinant RA33 protein used in this study, hence, consists of 314 amino acids (Fig. 1), all resembling the sequence of the human protein and yields in a calculated mass of 34,132 Da.

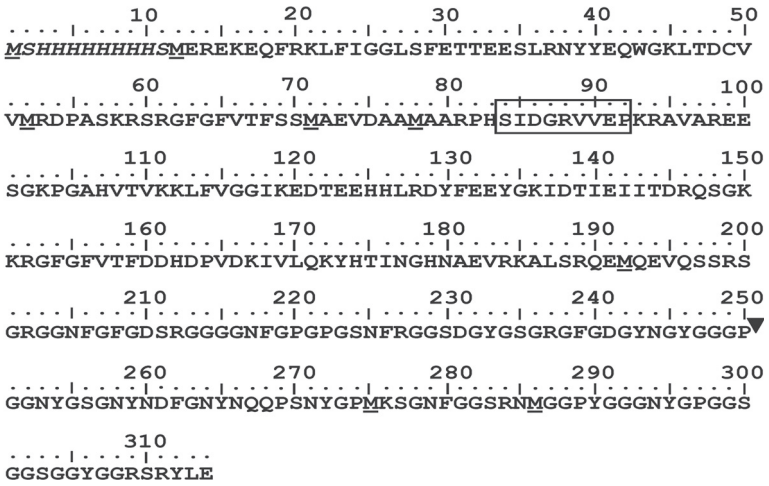

Figure 1: Amino acid sequence of recombinant RA33. The protein sequence resembles the A2 isoform of RA33 (Acc. No. P22626) with a His-tag at the N-terminus (printed in italic letters). The triangle maps the position, where the A2 isoform of RA33 bears an additional sequence stretch of 40 amino acids that is lacking in the recombinant RA33. Methionine residues are underlined. The boxed region contains the epitope that is recognized by the monoclonal antiRA33 antibody.

The protein sample reveals a strong band in SDS PAGE at apparent molecular mass of 33 kDa and other faint bands (truncated forms) at lower mass range (data not shown). Molecular mass determination of the HPLC-purified protein by MALDI ToF MS shows a singly charged molecular ion at *m/z* 34,166 and a small unassigned signal at *m/z* 22,812. This latter ion signal may result from a truncated RA33 form that has not been completely removed by HPLC (Fig. 2).

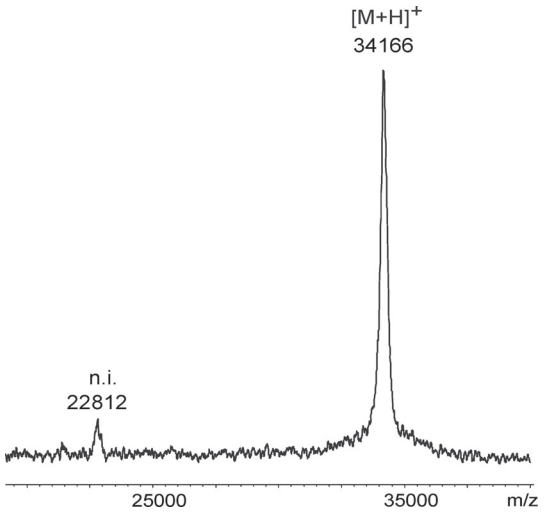

Figure 2: Linear MALDI ToF mass spectrum of HPLC purified recombinant RA33. A strong signal is observed for the singly charged molecular ion at *m/z* 34,166 (calculated Mr: 34,132). A small unidentified signal is seen at *m/z* 22,812 (n.i.: not identified). Matrix: Ferulic acid.

As the *C*-terminal end of the recombinant RA33 amino acid sequence was hardly determinable by peptide mass fingerprinting (data not shown), in-source decay (ISD) measurements were performed using the Reflex III MALDI ToF mass spectrometer. The mass range in which fragment ions were observed (*m/z* 1000–8000) showed a nearly complete partial sequence from aa 234-305 (Fig. 3). In the sequence gaps occurred at residues Asn253-Tyr254, at Pro269-Ser270, and at Gly298-Gly299-Ser300, respectively. This observation is consistent with previous ISD analysis of other proteins[19,20].

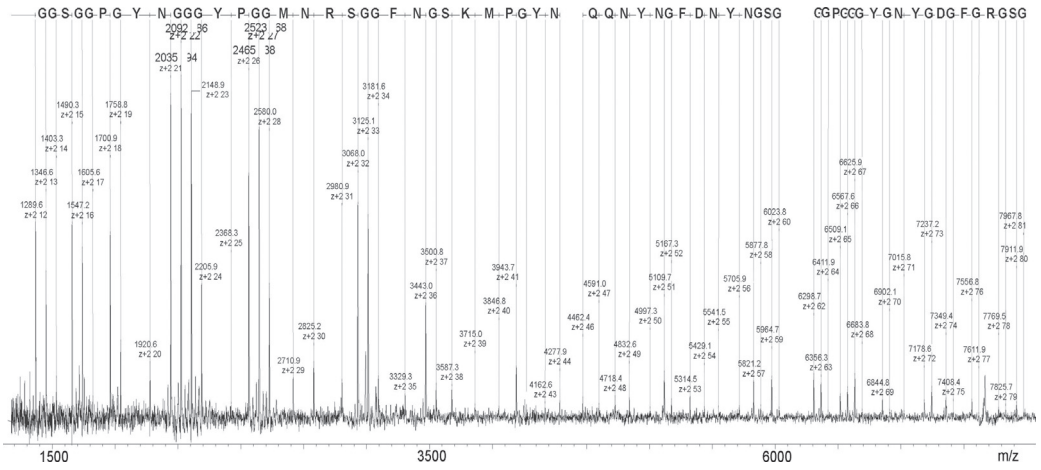

Figure 3: MALDI ToF ISD spectrum of HPLC purified recombinant RA33. Fragment ion signals are recorded in the mass range of *m/z* 1000 to 8000. The ion signals cover the *C*-terminal sequence from aa 234 to aa 305. In the sequence gaps occurred at residues Asn253-Tyr254 at Pro269-Ser270, and at Gly298-Gly299-Ser300, respectively. Z+2 ions are labeled. Matrix: DHB.

Combining all partial sequence information of peptide mapping experiments after digests using trypsin and Asp-N as well as the ISD measurements, we obtained sequence coverage of 98.4 % of the recombinant RA33 protein. Such high sequence coverage is presenting an appreciable starting position for assigning epitope regions as missing of ion signals for specific peptides in the mass spectrum certainly was disadvantageous for epitope determination, particularly when the epitope region was concerned.

4.2 BrCN degradation of recombinant RA33 and subsequent SDS PAGE and Western blot analysis of the fragment mixture with monoclonal antiRA33 antibody

HPLC purified recombinant RA33 was degraded by BrCN and the resulting fragment mixture showed no signals for intact recombinant RA33 in linear MALDI ToF mass spectra indicating complete degradation. Consistently, all

bands observed in SDS PAGE analysis of the BrCN-derived fragments were of lower apparent molecular masses than the intact full length RA33 mass (below 33 kDa; Fig. 4A, lane 2). Bands could be visualized down to an apparent mass of 3.4 kDa. However, below the 10 kDa mark bands were quite diffuse, making the estimation of apparent masses difficult. The MALDI ToF MS mixture analysis of the BrCN-derived fragments yielded in more precise mass estimations of the ion signals in the mass range between 2 and 10 kDa. All observed ion signals resulted in nearly complete sequence coverage of recombinant RA33 (Table 1). Fragments predominantly possessed *C*-terminal homo-Ser lactone residues.

Protein fragments containing bands 1 to 13 were blotted onto a PVDF membrane and subjected to Western blot analysis using a monoclonal antiRA33 antibody. Bands 1 to 9 were immuno-positive, thus, containing the epitope against which the monoclonal antiRA33 antibody is directed (Fig. 4B, lane 1). The smallest BrCN-derived fragment that stained positive was observed at apparent molecular mass of ca. 12 kDa (band 9 in Figs 4A and 4B). The largest non-immuno-positive band migrated with an apparent molecular mass of ca. 8 kDa (band 10 in Fig. 4A). Full-length RA33 protein (prior to HPLC) served as positive control, both, for SDS PAGE (Fig. 4A, lane 1) and for Western blot analysis (Fig. 4B, lane 2). As has been described above, non-HPLC purified samples of RA33 showed a strong band at an apparent mass of 33 kDa accompanied with faint bands for truncated forms. These truncated forms of RA33 also stained with the monoclonal antiRA33 antibody in the Western blot analysis.

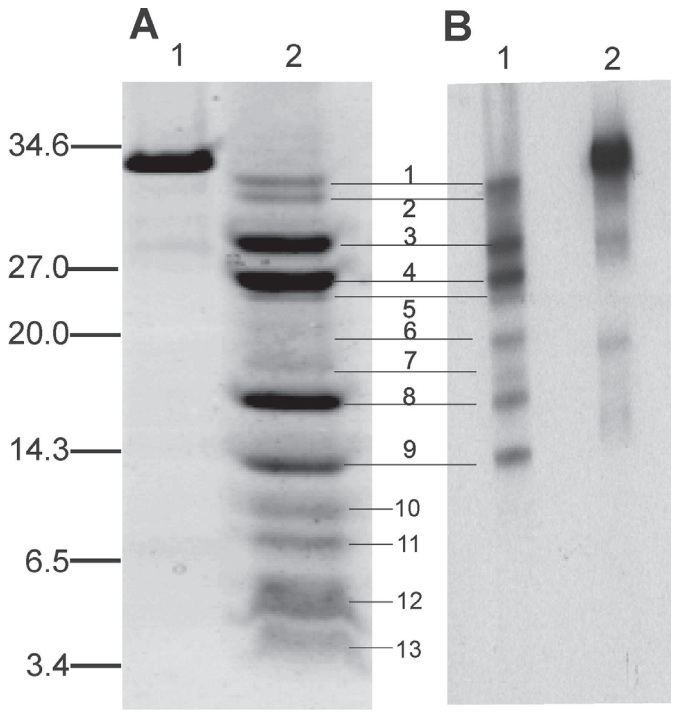

Figure 4: SDS PAGE and Western Blot analysis of BrCN-derived fragments of recombinant RA33. The SDS PAGE image is aligned to the matching Western Blot image. **A:** 1D SDS PAGE. Lane 1: Full-length RA33. Lane 2: Bands of BrCN-derived fragments of RA33. **B:** Western Blot analysis. Lane 1: Immuno-positive bands due to the reaction of the BrCN-derived fragments with the monoclonal antiRA33 antibody. Lane 2: Full length protein. Bands of BrCN-derived fragments are numbered beginning from the top. Numbering of immuno-positive bands is printed in bold letters. Molecular mass marker positions are indicated on the left (numbers refer to apparent masses in kDa). For partial sequence assignments of BrCN-derived fragments of recombinant RA33 see Table 2.

In-gel trypsin digest of the BrCN-derived fragments in bands 1 to 13 from the Coomassie-stained SDS gel allowed estimation of sequence ranges of the respective BrCN-derived fragments; at least of the dominant fractions in a band when several fragments with similar masses overlapped (Supplemental Table 1). In addition to the sequence coverage, our special interest was focused on the determination of possible end-groups of BrCN-derived fragments. Guided by the mixture analysis (Table 1), we searched for such peptides which were cleaved by BrCN on the one side and by trypsin on the other. Using this information on identified end groups together with the determined apparent molecular masses from the SDS PAGE runs enabled the estimation of the partial sequence ranges of the BrCN-derived fragment(s) in each band.

Table 1: MALDI MS analysis of BrCN-derived fragment mixture of RA33.

Sequence Range	Exp. Mass (*m/z*)	Calc. Mass $[M+H]^+$; (Da)
13 - 52	4814 [a,b)]	4814.5 [c)]
53 - 71	2085.52	2085.06 [c)]
55 - 71	1813.34	1813.93 [c,d)]
53 - 78	2790.28	2790.35 [e)]
55 - 78	2519.17	2519.22 [d,e)]
79 - 163	9620 [a)]	9622.7 [d)]
164 - 192	3326.63	3326.80 [c,d)]
193 - 275	8212.5 [a,b)]	8212.5 [c,f)]
276 - 286	1106.76	1106.53 [c)]
287 - 314	2593.67	2593.15

a) linear ToF mass spectrum
b) used for internal calibration
c) *C*-terminal homo-Ser lactone
d) mild acid cleavage of Asp-Pro bond
e) *C*-terminal homo-Ser
f) *N*-terminal pyro-Glu

Next, we were checking if the internal tryptic peptides that were recorded in the peptide mass fingerprints were all falling within the estimated sequence ranges in order to substantiate the sequence assignments. As an example, the ion signal at *m/z* 1079.33 was assigned to belong to peptide aa 79-88 (Fig. 5). This peptide was cleaved by BrCN at Met78 and by trypsin at Arg88, and therefore marked the N-terminus of a BrCN-derived fragment with an apparent mass of ca. 15 kDa (band 8; *cf*. Supplemental Table 1) which, consequently, had to extend to a residue beyond Met192 but had to end before Met275. Consistent with this rationing, the most *C*-terminal peptide that could be assigned was the tryptic peptide ranging from aa 213-227 (*m/z* 1377.31). All other tryptic peptides were within the sequence borders aa 79-227. Such a fragment may have arisen by the BrCN fragmentation of a truncated RA33 that was present even after HPLC purification. The precise *C*-terminal end of this fragment, however, remains unassigned.

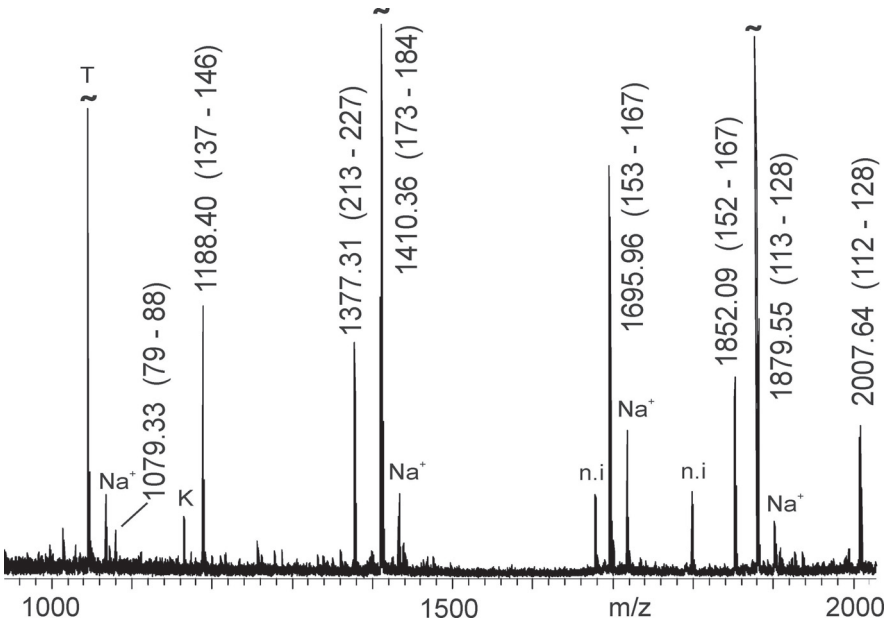

Figure 5: MALDI ToF MS peptide mapping analysis after *in-gel* trypsin digest of BrCN-derived protein fragments from immuno-positive band 8 of the SDS PAGE analysis (*cf.* Fig. 4). Peptide ion signals are labeled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses. T indicates ion signals due to trypsin auto-proteolysis. Matrix: CHCA.

Comparably, in band 9 an ion signal at *m/z* 1256.64 marked a peptide that had been cleaved after Arg 153 on one side and after Asp 163 (by so-called mild acid cleavage) on the other. Mild acid cleavage is a side reaction that takes place during BrCN degradation predominantly at Asp-Pro bonds. Hence, this peptide marks a *C*-terminal end of a 12 kDa fragment which, therefore, had to start most likely at Pro 55. Pro 55 also marks a "mild acid" cleavage site. The calculated average molecular mass for this fragment aa 55-163 is 12,153.6 Da. With the same reasoning, BrCN-derived fragments aa 79-192 (M_r (avg.): 12,931.6) and aa 13-121 (M_r (avg.): 12,239.9) were assumed to be co-migrating in this band. As this band was the lightest immuno-positive band, the epitope had to be within one of these assigned fragments.

Similar to the reasoning described above, the sequence range of aa 1-71 was assigned to the heaviest band that was immuno-negative (band 10; Fig. 4), excluding the *N*-terminal region as epitope carrying range of RA33. Also, as immuno-negative band 11 (apparent molecular mass: 6 kDa, starting with aa 164) contained a peptide ion signal for aa 173-184 (*m/z* 1410), the eptiope region in recombinant RA33 was estimated to be situated *N*-terminally to the amino acid residue 163. Taking all information on identified partial sequences in all SDS PAGE bands together and aligning this information with the immuno-

positive fractions from the Western blot analysis (Fig. 4), we deduced the epitope region against which the monoclonal anti RA33 antibody is directed to be within partial amino acid sequence aa 79-163 of recombinant RA33.

4.3 BrCN degradation of recombinant RA33, subsequent HPLC, and dot blot analysis of the fragment mixture with the monoclonal antiRA33 antibody

BrCN-derived RA33 fragments were separated by HPLC yielding in ten major fractions that were collected individually (Fig. 6A). Sharp HPLC peaks were obtained for early eluting fractions. By contrast, later eluting fractions (*e.g.* fractions 8 and 9) were forming fairly broad signals in the chromatogram, indicating that the respective RA33 fragments were eluting from the column over an extended period of time.

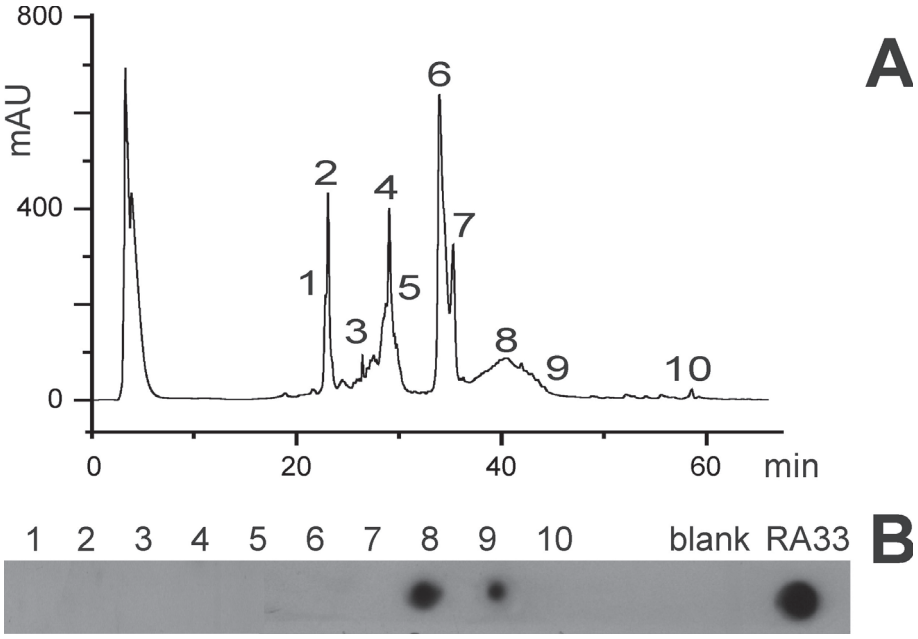

Figure 6: HPLC and dot blot analysis of BrCN-derived fragments of recombinant RA33. **A:** HPLC chromatogram of protein fragments after BrCN degradation. Numbers indicate major fractions that were collected and subjected to mass spectrometric analysis and to dot blot analysis, respectively. Detection wavelength: 280 nm. For partial sequence assignments of BrCN-derived fragments of recombinant RA33 see Table 3. **B:** Dot blot analysis of the HPLC separated fractions and a blank (negative control) as well as the full-length RA33 (positive control).

Despite their low intensities in the chromatogram, the amount of protein that eluted in these fractions was quite significant. Dot blot analysis of all HPLC fractions (Fig. 6B) showed that only the BrCN-derived RA33 fragments from fractions 8 and 9 were immuno-positive.

Mass spectrometric analysis of all HPLC fractions enabled assignment of BrCN-derived RA33 fragments (Table 2). As an example, the linear MALDI-ToF mass spectrum of fragments in HPLC fraction 8 showed strong signals in the mass range of *m/z* 3,000-13,500 (Fig. 7).

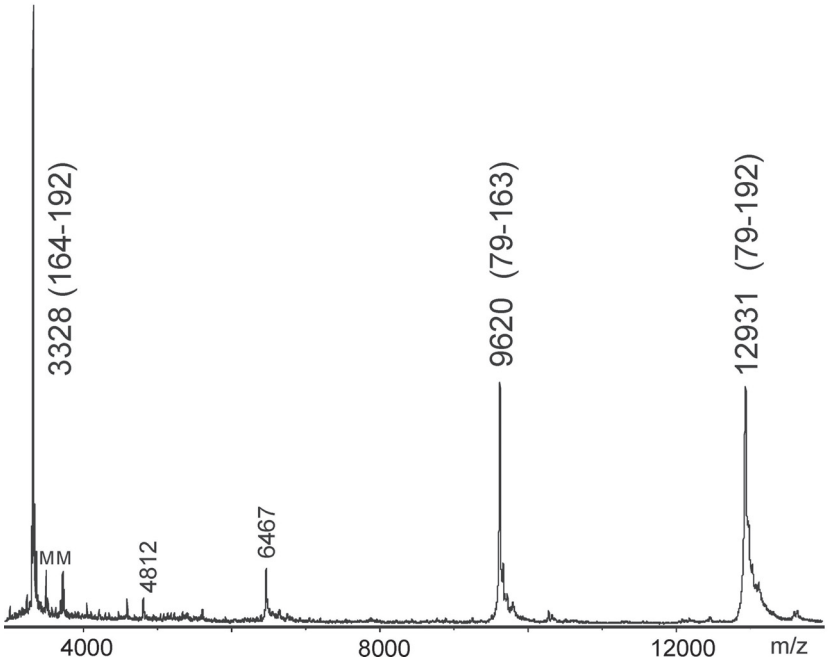

Figure 7: MALDI ToF MS analysis of a protein fragment from immuno-positive HPLC fraction 8 (*cf.* Figure 6). The spectrum was recorded in linear mode (*m/z*: 3000–35,000). The region from *m/z* 3000–14000 is shown. Peptide ion signals are labelled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses. For complete assignments *cf.* Table 3. M indicates matrix adducts. Matrix: DHB.

The ion signal at *m/z* 12,931 was assigned to the partial sequence aa 79-192 (calculated: 12,931.6 Da) and the ion signal at *m/z* 9620 to the partial sequence aa 79-163 (calculated: 9621.7 Da). The matching doubly charged ion signals were observed at *m/z* 6467 and *m/z* 4812, respectively. Another strong ion signal at *m/z* 3329 matched with amino acid sequence aa 164-192 (calculated: 3326.80 Da), showing that peptides with partial sequences starting from aa 79 up to aa 192 co-eluted in this fraction.

Table 2: MALDI MS analysis of RA33 fragments after BrCN cleavage and HPLC separation.

Fraction No.	Exp. Mass (*m/z*)	Calc. Mass $[M+H]^+$; (Da)	Sequence Range
1	1503.67	1503.63 [b)]	1–12
2	2593.27	2593.15	287–314
	3700.60	3700.90 [c)]	276–314
3	2593.10	2593.15	287-314
	3701 [a)]	3700.9 [c)]	276–314
	3747 [a)]	3747.9 [d)]	276–314
4	3326.12	3326.80 [b,e)]	164–192
	3346 [a)]	3346.9 [e,f)]	164–192
5	5894 [a)]	-	- [g)]
	5949 [a)]	-	- [g)]
	6006 [a)]	-	- [g)]
	6063 [a)]	-	- [g)]
6	2085.64	2085.06 [b)]	53–71
	2470.75	2471.2 [b,c,e,)]	55–78
	2789.71	2790.35 [f)]	53–78
	3327 [a)]	3326.8 [b,e)]	164–192
	8229 [a)]	8230.5 [b)]	193–275
	9318 [a)]	9318.7 [b,c,h)]	193–286
7	2085.70	2085.06 [b)]	53–71
	2518.25	2519.17 [e,f)]	55–78
	2789.04	2790.35 [f)]	53–78
	8212 [a)]	8212.5 [b,h)]	193–275
8	2085.70	2085.06 [b)]	53–71
	3326.45	3326.80 [b,e)]	164–192
	3344.33	3344.80 [e,f)]	164–192
	9620 [a)]	9622.7 [e)]	79–163
	12931 [a)]	12931.6 [b)]	79–192
	10279 [a)]	10280.5 [c,e)]	72–163
	10324 [a)]	10326.5 [d,e)]	72–163
	13636 [a)]	13638.4 [f)]	72–192
	13590 [a)]	13590.4 [c,f)]	72–192
9	4812 [a)]	4814.5 [b)]	13–52
	6343 [a)]	6348.1 [b)]	1–52

a) linear ToF mass spectrum

b) *C*-terminal homo-Ser lactone

c) conversion of internal Met residue to homo-Ser without cleavage

d) oxidation of internal Met residue

e) mild acid cleavage of Asp-Pro bond

f) *C*-terminal homo-Ser

g) not assigned

h) *N*-terminal pyro-Glu

Taking all information on identified partial sequences in all HPLC fractions together (Table 2) and aligning this information with the immuno-positive fractions from the dot blot analysis (Fig. 6B), we deduced that the epitope region against which the monoclonal antiRA33 antibody is directed to be within

the partial amino acid sequence aa 79-163 of the recombinant RA33. This result is matching exactly with that from the epitope mapping experiments coming from SDS PAGE in combination with Western blot analysis (see above).

4.4 Peptide chip analysis of synthetic RA33 peptides with monoclonal antiRA33 antibody and epitope assignment

Peptide chips contained synthetic 15-mer peptides of RA33 isoforms listed as entries P22626, P09651, and Q99729 in the UniProt database (NP_002128 and NP_112533 in NCBI RefSeq). A frame-shift of 6 amino acid residues was chosen, resulting in 59 peptides by which the full-length protein sequences were represented on the chip surface without redundancies. The monoclonal antiRA33 antibody used in this study showed strong signals with mean values above 10,000 units for 15 of the 9176 individual peptides on the chip (Table 3).

Table 3: Epitope motif alignment of 15-mer peptides probed on chip with monoclonal antiRA33 antibody.

No.	15-mer Peptide Sequence [a,b,c]	UniProt [d]	Peptide Chip Signal [e]
1	KDSRA.IIRENEFSFE	Q12873	51616
2	VTYTGDKDSRA.IIRE	Q12873	31042
3	DSRA.IIRENEFSFED	Q12873	13758
4	TYVGDKDSRA.IIREN	Q14839	15760
5	LLESYIDGR..IV.EGSD	P00734	46237
6	MAARPHSIDGR...VVEP	P22626	36655
7	SIDGR...VVEPKRAVAR	P22626	10355
8	TFGCDGRMD..SQQVWD	Q76LX8	39532
9	EQFLDGDGWTSR..WI.E	P27797	42260
10	DGWTSR..WI.ESKHKSD	P27797	40728
11	MDSYSRFEIVGENTR	Q13247	21138
12 [f]	STR.P...EKFLGDIEVWD	P26639	16094
13 [f]	STRPEKFLGDI.EVWD	P26639	16094
14	YGP.P.I.ENGFYYDMYL	P26639	16272
15	YGGCLCYGP.P.I.ENGF	P26639	10885
16	WGMEYKGYLVSV.DGY	P62306	60143

a) GLAM2 program: http://bioinformatics.org.au/glam2/

b) Residues printed in bold are part of the consensus sequence

c) Underlined sequences are from RA33

d) Protein accession number from UniProt data base

e) Mean values from triplicate measurements

f) two alternative alignments for the same 15-mer sequence

Within these 15 best scoring peptides two were from RA33. The GLAM2 algorithm[21] was applied to search for common motives in the 14 sequences.

The best matching motif that coincides on recombinant RA33 with high homology to the other sequences that are immuno-positive on the chip is DGRVVE.

In fact, the sequence motif analysis suggests that the epitope peptide consists of three parts The *N*-terminal partial motif consists of three amino acids that may be described as “acidic/polar–small–basic” which is best represented by the partial sequence “DSR”. The *C*-terminal partial motif is more promiscuous and contains the properties “hydrophobic–gap–acidic”. This motif is best represented by the partial sequence “I_E”. The gap between these partial motives is up to three amino acids in length. The resulting consensus sequence is “DSR (_) I_E”. The consensus peptide is between 7 to 9 amino acids long, matches with known sizes of B-cell epitopes.

The 15mer peptide that belonged to the RA33 sequence with the highest value in the chip measurements was covering the range from aa 78-92 (MAARPHSIDGRVVEP: signal intensity 36,655; Table 4), followed by a peptide which covered the adjacent *C*-terminal sequence range aa 84-98 (SIDGRVVEPKRAVAR: signal intensity 10,355). Both peptides share the 9 amino acid long sequence SIDGRVVEP.

Table 4: Epitope peptide chip results.

Sequence Range	Partial Sequence	Mean Signal intensity	SD
78-92	MAARPHSIDGRVVEP	36655	13078
84-98	SIDGRVVEPKRAVAR	10355	2409
72-86	AEVDAAMAARPHSID	1684	264

It should be noted that the underlined RA33 partial sequence correlates very well with the consensus sequence “DSR (_) I_E”. The peptide that is recognized as third best from the RA33 sequence on the chip surface is *N*-terminally shifted (range aa 72-86) and does not contain the amino acids from the consensus sequence. The recorded signal for this peptide is, accordingly, much lower (signal intensity 1684). All other peptides from the RA33 sequence on the chip resulted in even lower signals in the chip measurements. Hence, it can be concluded that the epitope for the monoclonal antiRA33 antibody is consisting of the amino acid residues SIDGRVVEP that comprise the range aa 84-92 of RA33. This result is well in agreement with the BrCN fragmentation experiments described above.

5. Discussion

It has been predicted that in the coming decades, proteomics technologies will broaden our understanding of the underlying mechanisms of the roles of autoantigens in autoimmune diseases and will further our ability to diagnose, prognosticate and treat such autoimmune diseases[22]. Common knowledge states that epitopes on proteins were mostly conformational[23]. However, translating the information on conformational epitopes into methods for screening of patients in a “cost-effective” manner has been found complicated. Whole protein microarrays have still been difficult to produce and to handle[10,24]. Clearly, the use of peptide chips is more robust than using chips with immobilized proteins and provides several advantages for clinical purposes. Peptides are easily synthesized in a standardized, reproducible, and cost-effective manner, they can be covalently and specifically attached (including spacers or any chemical modification) with nearly equal number of molecules per spot, and peptide microarrays are rather stable. Hence, for diagnostic and prognostic means it seems more suitable to focus on “peptide-sized” epitopes that are recognized by autoantibodies from patients[10], whenever this was possible. One prime example for peptide-based diagnostics is the use of so-called “CCP-assays” (cyclic citrullinated peptides) for diagnosing RA[25].

The epitope mapping approach described here is demonstrating that epitope sequences which are detected by peptide chip analysis can also be determined on full-length proteins. While this seems trivial to state it is important to demonstrate this fact experimentally, as autoantibody profiling through epitope peptide chip analysis relies on the correlation between screening results and the pathological situation of an individual. Without analyzing the epitope on the full-length protein the use of peptide chips bears the general problem of potentially studying so-called "cryptic" epitopes. Cryptic epitopes are antibody-binding regions that appear as binding surfaces when partial sequences and not the full-length proteins are exposed to antibodies. These antibody bindings have to be distinguished from cross-reactivities[26,27] that may occur when antibodies bind to other full-length proteins, leading to false positive results. Obviously, epitope determination on the full-length antigen is required and powerful mass spectrometric epitope mapping approaches for analyzing antibody binding structures including conformational epitopes have been described. Mass spectrometry-based epitope identification methods involve epitope excision[28-33] and epitope extraction[34-39]. This set of methods may be summarized, together with methods in which immunosorption steps have been integrated into mass spectrometric analysis as “affinity mass spectrometry”[40-42]. Epitope extraction is

the proteolytic digestion of the free antigen and presentation of the mixture of peptide fragments to the antibody (mostly a monoclonal antibody), followed by isolation of the antibody-peptide complex. The epitope and non-epitope fractions are analyzed by mass spectrometry, and the epitope sequences are identified directly. In our experiments the mass spectrometrically determined epitope-carrying peptides where ca. 80 aa residues in length and as such too large for describing the binding surface only, which for antibodies is estimated to be ca. 10 to 12 aa. Several reasons may cause loss of signal intensity by Western blot and also to dot-blot analyses. Short peptides are difficult to capture on blot membranes and, therefore, may escape detection. Also, cleavage of the epitope peptide at the binding site may also cause loss of recognition when the epitope is split into two halves. In order to avoid these expected adverse effects, we decided to use BrCN for cleavage. BrCN leads to larger protein fragments as the distribution of methionine residues in general is scarce as compared to that of *e.g.* lysine and arginine residues. The lack of spatial resolution was compensated by adding peptide chip analysis.

As mentioned, epitope profiling should ultimately serve important clinical purposes including classification of individual patients and determining subsets of patients based on their “epitope profile”, examination of epitope spreading and antibody isotype usage, discovery and characterization of candidate autoantigens, and tailoring antigen-driven therapy. The fact that even a monoclonal antibody can give rise to multiple signals on a peptide chip underlines the importance of thorough studies on epitope structures / sequences and binding interactions between (auto)antibodies and (auto)antigens. Cross-reactivities of antibodies may be important with respect to disease development.

As can be seen in the 3D structure model of recombinant RA33$^{43\text{-}45}$, the epitope which is recognized by the monoclonal antibody is exposed on the surface of the protein (Fig. 8). As the part of the protein where the epitope is located has been crystallized (PDB structure: 1HA1; hnRNP A1) and also has been studied by NMR (PDB structure: 1X4B; solution structure of domain I in hnRNP A2/B1), we can visualize the epitope as a β-sheet–hairpin–β-sheet structure with high accuracy. As of yet the 3D structure of the full-length RA33 protein has not been published.

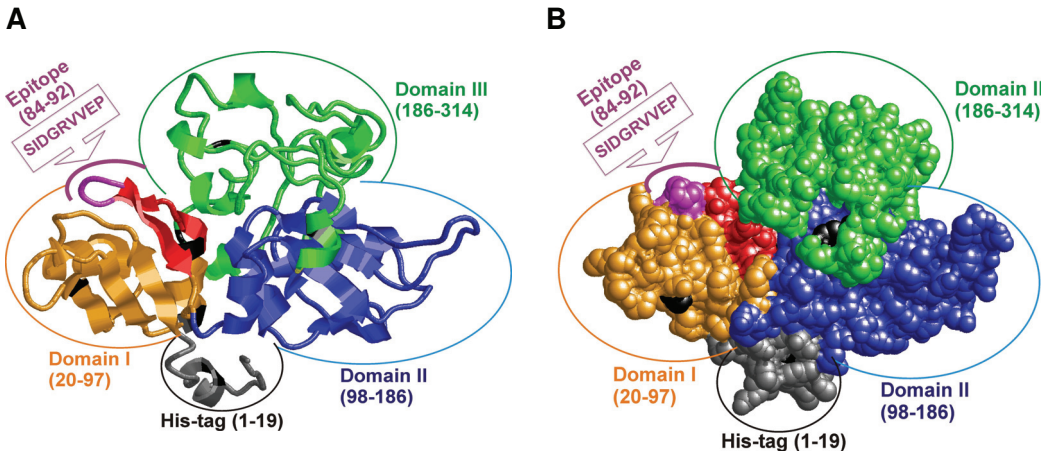

Figure 8: Structural model of recombinant RA33. The three-dimensional structure representation has been modeled using the "I-TASSER" algorithm (http://zhang.bioinformatics.ku.edu/I-TASSER). **A**: The ribbon diagram shows β-sheet (arrows), α-helices (spirals), and loops (threads). **B**: The molecular surface is indicated with van-der-Waals spheres. The three domains and the *N*-terminal His-tag are circled. Sequence ranges are given in parentheses. Residues shown in black mark Methionines. The arrow points to the epitope region on the surface of the RA33 structure.

When analyzing the partial sequences from the 15 best scoring peptides obtained by peptide chip analysis, the gap between the here described partial motives "DSR" and "I_E" was in most cases up to three amino acids in length. However, in one case (entries 12 and 13 in Table 3) there are indications that there might even be a larger loop possible between the antibody binding spots presented on a 15-meric peptide. The binding motif: –**STR**-(PEKFLGD)-**I_E**-VWD would be a matching alternatively to the listed binding motives. Whether such a binding conformation was really occurring during the chip analysis - perhaps as a result of so-called "induced fit" - or if it was present in a mixed situation with other possible (weaker) binding structures remains an open question. In general, it seems difficult to define a binding motif (the epitope) as being strictly "linear" in almost any case.

Concerning our studies it is of interest that antiRA33 autoantibodies may appear shortly after the onset of rheumatoid arthritis before a final diagnosis can be made. Therefore, they can provide diagnostic help in early disease, particularly when the RF is negative. In a single case, the presence of the antiRA33 antibody even preceded disease symptoms by approximately one year[5]. The importance of RA33 in the development of RA and associated disease models has been substantiated in recent animal model studies that have shown the increase of hnRNP A2 (RA33) abundance in joints of animals with erosive

arthritis as a result of increased TNF alpha expression. This overexpression of hnRNP A2 correlated with the presence of antiRA33 autoantibodies in these animals[46], suggesting that the expression of hnRNP A2/B1 and/or the presence of the autoantibodies directed against this protein may enhance the inflammatory and destructive process. In agreement with this assumption it was reported that pristane induced arthritis (PIA) in rats resulted in increase of antiRA33 autoantibodies, here preceding onset of the disease[47]. Again it has been suggested that hnRNP A2/B1 is among the primary inducers of autoimmunity in PIA, and possibly in RA.

In conclusion, as autoimmune diseases are chronic inflammatory diseases whose development, progression, and recurrence are frequently difficult to diagnose[48], improving our knowledge in diagnostic or prognostic procedures constitutes a research and development area of strategic importance.

6. Acknowledgements

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7. References

1. J. Avouac, L. Gossec and M. Dougados, "Diagnostic and predictive value of anti-CCP (cyclic citrullinated protein) antibodies in rheumatoid arthritis: a systematic literature review", Ann. Rheum. Dis. 65, 845 (2006). doi: 10.1136/ard.2006.051391

2. S. Drynda, B. Ringel, M. Kekow, C. Kühne, A. Drynda, M.O. Glocker and H.-J. Thiesen, "Proteome analysis reveals disease-associated marker proteins to differentiate RA patients from other inflammatory joint diseases with the potenial to monitor anti-TNFa therapy", Pathol. Res. Pract. 200, 165 (2004). doi: 10.1016/j.prp.2004.02.011

3. M.O. Glocker, R. Guthke, J. Kekow and H.-J. Thiesen, "Rheumatoid arthritis, a complex multifactorial disease: on the way toward individualized medicine", Med. Res. Rev. 26, 63 (2006). doi: 10.1002/med.20045

4. J.R. Kirwan and B. Quilty, "Prognostic criteria in rheumatoid arthritis: can we predict which patients will require specific anti-rheumatoid treatment?", Clin. Exp. Rheumatol. 15, S15 (1997).

5. G. Steiner, K. Skriner, W. Hassfeld and J.S. Smolen, "Clinical and immunological aspects of autoantibodies to RA33/hnRNP-A/B proteins-a link between RA, SLE and MCTD", Mol. Biol. Rep. 23, 167 (1996). doi: 10.1007/BF00351165

6. G. Steiner and J.S. Smolen, "Neue Autoantikörper in der Diagnostik der rheumatoiden Arthritis", Z. Rheumatol. 61, 667 (2002). doi: 10.1007/s00393-002-0470-y

7. E. Feist, K. Egerer and G.-R. Burmester, "Autoantikörperprofile bei der rheumatoiden Arthritis", Z. Rheumatol. 66, 212 (2007). doi: 10.1007/s00393-007-0159-3

8. C.S. Raska, C.E. Parker, S.W. Sunnarborg, R.M. Pope, D.C. Lee, G.L. Glish and C.H. Borchers, "Rapid and sensitive identification of epitope-containing peptides by direct matrix-assisted laser desorption/ionization tandem mass spectrometry of peptides affinity-bound to antibody beads", J. Am. Soc. Mass Spectrom. 14, 1076 (2003). doi: 10.1016/S1044-0305(03)00405-7

9. S. Dhungana, J.G. Williams, M.B. Fessler and K.B. Tomer, in: Epitope Mapping Protocols; M. Schutkowski and U. Reineke, Eds., Humana Press, Vol. 524, pp 87 (2007).

10. P. Lorenz, M. Kreutzer, J. Zerweck, M. Schutkowski and H.-J. Thiesen, in: Meth. Mol. Biol. Epitope Mapping Protocols; U. Reineke and M. Schutkowski, Eds., Humana Press, Vol. 524, pp 247 (2009).

11. M.O. Glocker, B. Arbogast, J. Schreurs and M.L. Deinzer, "Assignment of the inter- and intramolecular disulfide linkages in recombinant human macrophage colony stimulating factor using fast atom bombardment mass spectrometry", Biochemistry 32, 482 (1993). doi: 10.1021/bi00053a012

12. V. Neuhoff, N. Arold, D. Taube and W. Ehrhardt, "Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250", Electrophoresis 9, 255 (1988). doi: 10.1002/elps.1150090603

13. J.C. Heitner, C. Koy, T. Reimer, M. Kreutzer, B. Gerber and M.O. Glocker, "Differentiation of HELLP Patients from Healthy Pregnant Women by Proteome Analysis", J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 840, 10 (2006). doi: doi:10.1016/j.jchromb.2006.06.002

14. M. Kienbaum, C. Koy, H.V. Montgomery, S. Drynda, P. Lorenz, H. Illges, K. Tanaka, J. Kekow, R. Guthke, H.-J. Thiesen and M.O. Glocker "Mass Spectrometric Characterization of Apheresis Samples from Rheumatoid Arthritis Patients for the Improvement of Immunoadsorption Therapy-A Pilot Study", Proteomics Clin. Appl. 3, 797 (2009).

15. T. Köcher, Å. Engström and R.A. Zubarev, "Fragmentation of Peptides in MALDI In-Source Decay Mediated by Hydrogen Radicals", Anal. Chem. 77, 172 (2005). doi: 10.1021/ac0489115

16. D. Suckau and A. Resemann, "T3-Sequencing: Targeted Characterization of the N- and C-Termini of Undigested Proteins by Mass Spectrometry", Anal. Chem. 75, 5817 (2003). doi: 10.1021/ac034362b

17. C. Koy, S. Mikkat, E. Raptakis, C. Sutton, M. Resch, K. Tanaka and M.O. Glocker, "Matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight mass spectrometry sequencing resolves structures of unidentified peptides obtained by in-gel tryptic digestion of haptoglobin derivatives from human plasma proteomes", Proteomics 3, 851 (2003). doi: 10.1002/pmic.200300381

18. C. Koy, M. Resch, K. Tanaka and M.O. Glocker, "Primary structure details of haptoglobin a chain proteins from human plasma samples are resolved by QIT ToF MSn sequencing", Eur. J. Mass Spectrom. 10, 393 (2004). doi: 10.1255/ejms

19. D. Suckau and D.S. Cornett, "Protein sequencing by ISD and PSD MALDI-TOF MS", Analusis Mag. 26, M18 (1998). doi: 10.1051/analusis:1998260018

20. M. Takayama and A. Tsugita, "Sequence information of peptides and proteins with in-source decay in matrix assisted laser desorption/ionization-time of flight-mass spectrometry", Electrophoresis 21, 1670 (2000). doi: 10.1002/(SICI)1522-2683(20000501)21:9<1670::AID-ELPS1670>3.0.CO;2-N

21. M.C. Frith, N.F.W. Saunders, B. Kobe and T.L. Bailey, "Discovering sequence motifs with arbitrary insertions and deletions", PLoS Computational Biology", PLoS Comput. Biol. 4, e1000071 (2008). doi: doi/10.1371/journal.pcbi.1000071

22. W. Hueber, P.J. Utz, L. Steinman and W. Robinson, H., "Autoantibody profiling for the study and treatment of autoimmune disease", Arthritis Res. 4, 290 (2002). doi:

23. D.J. Barlow, M.S. Edwards and J.M. Thornton, "Continuous and discontinuous protein antigenic determinants", Nature 322, 747 (1986). doi: 10.1038/322747a0

24. M. Cretich, F. Damin, G. Pirri and M. Chiari, "Protein and peptide arrays: recent trends and new directions", Biomol. Eng. 23, 77 (2006). doi: 10.1016/j.bioeng.2006.02.001

25. L. Klareskog, J. Rönnelid, K. Lundberg, L. Padyukov and L. Alfredsson, "Immunity to citrullinated proteins in rheumatoid arthritis", Annu. Rev. Immunol. 26, 651 (2008). doi: 10.1146/annurev.immunol.26.021607.090244

26. M.A. Fouraux, A. van der Heijden, W.J. van Venrooij and G.J.M. Pruijn, "Cross-reactivity of the anti-La monoclonal antibody SW5 with early endosome antigen 2", Immunology 106, 336 (2002). doi: 10.1046/j.1365-2567.2002.01432.x

27. H. Hasegawa, T. Uchiumi, T. Sato, M. Arakawa and R. Kominami, "Anti-Sm autoantibodies cross-react with ribosomal protein S10: a common structural unit shared by the small nuclear RNP proteins and the ribosomal protein", Arthritis Rheum. 41, 1040 (1998). doi: 10.1002/1529-

0131(199806)41:6<1040::AID-ART10>3.0.CO;2-2

28. C. Hager-Braun, H. Katinger and K.B. Tomer, "The HIV-neutralizing monoclonal antibody 4E10 recognizes N-terminal sequences on the native antigen", J. Immunol. 176, 7471 (2006).

29. E.O. Hochleitner, C. Borchers, C. Parker, R.J. Bienstock and K.B. Tomer, "Characterization of a discontinuous epitope of the human immunodeficiency virus (HIV) core protein p24 by epitope excision and differential chemical modification followed by mass spectrometric peptide mapping analysis", Protein Sci. 9, 487 (2000).

30. S. Jeyarajah, C.E. Parker, M.T. Sumner and K.B. Tomer In J. Am. Soc. Mass Spectrom., 1998; Vol. 9, pp 157.

31. P. Juszczyk, G. Paraschiv, A. Szymanska, A.S. Kolodziejczyk, S. Rodziewicz-Motowidlo, Z. Grzonka and M. Przybylski, "Binding epitopes and interaction structure of the neuroprotective protease inhibitor cystatin C with beta-amyloid revealed by proteolytic excision mass spectrometry and molecular docking simulation", J. Med. Chem. 52, 2420 (2009). doi: 10.1021/jm801115e

32. M. Macht, W. Fiedler, K. Kürzinger and M. Przybylski, "Mass spectrometric mapping of protein epitope structures of myocardial infarct markers myoglobin and troponin T", Biochemistry 35, 15633 (1996). doi: 10.1021/bi961727w

33. R. Stefanescu, R.E. Iacob, E.N. Damoc, A. Marquardt, E. Amstalden, M. Manea, I. Perdivara, M. Maftei, G. Paraschiv and M. Przybylski, "Mass spectrometric approaches for elucidation of antigenantibody recognition structures in molecular immunology", Eur. J. Mass Spectrom. 13, 69 (2007). doi: 10.1255/ejms.849

34. Z. Bílková, R. Stefanescu, R. Cecal, R. Korecká, Š. Ouzká, J. Ježová, J.-L. Viovya and M. Przybylski, "Epitope extraction technique using a proteolytic magnetic reactor combined with Fourier-transform ion cyclotron resonance mass spectrometry as a tool for the screening of potential vaccine lead peptides", Eur. J. Mass Spectrom. 11, 489 (2005). doi: 10.1255/ejms.782

35. A. Czogalla, P. Kwoleka, A. Hryniewicz-Jankowska, N. Małgorzata, L. Jacek and A.F. Sikorski, "A protein isolated from Escherichia coli, identified as GroEL, reacts with anti-beta spectrin antibodies", Arch. Biochem. Biophys. 415, 94 (2003). doi: 10.1016/S0003-9861(03)00223-6

36. N. Sato, Y. Nabeta, H. Kondo, H. Sahara, Y. Hirohashi, K. Kashiwagi, T. Kanaseki, Y. Sato, S. Rong, I. Hirai, K. Kamiguchi, Y. Tamura, A. Matsuura, S. Takahashi, T. Torigoe and H. Ikeda, "Human CD8 and CD4 T cell epitopes of epithelial cancer antigens", Cancer Chemother. Pharmacol. 46, S86 (2000). doi:

37. D. Suckau, J. Kohl, G. Karwath, K. Schneider, M. Casaretto, D. Bitter-Suermann and M. Przybylski, "Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping", Proc. Natl. Acad. Sci. 87, 9848 (1990).

38. X. Tian, M. Maftei, M. Kohlmann, B. Allinquant and M. Przybylski, in: Subcellular Biochemistry; E. Bertrand and M. Faupel, Eds., Springer, The Netherlands, Vol. 43, pp 339 (2007).

39. L. Yu, S.J. Gaskell and J.L. Brookman, "Epitope mapping of monoclonal antibodies by mass spectrometry: identification of protein antigens in complex biological systems", J. Am. Soc.

Mass Spectrom. 9, 208 (1998). doi: doi:10.1016/S1044-0305(97)00250-X

40. M. Macht, A. Marquardt, S.-O. Deininger, E. Damoc, M. Kohlmann and M. Przybylski, ""Affinity-proteomics": direct protein identification from biological material using mass spectrometric epitope mapping", Anal. Bioanal. Chem. 378, 1102 (2004). doi: 10.1007/s00216-003-2159-8

41. O. Poetz, S. Hoeppe, M.F. Templin, D. Stoll and T.O. Joos, "Proteome wide screening using peptide affinity capture", Proteomics 9, 1518 (2009). doi: 10.1002/pmic.200800842

42. C. Wingren, P. James and C.A.K. Borrebaeck, "Strategy for surveying the proteome using affinity proteomics and mass spectrometry", Proteomics 9, 1511 (2009). doi: 10.1002/pmic.200800802

43. S. Wu, J. Skolnick and Y. Zhang, "Ab initio modeling of small proteins by iterative TASSER simulations", BMC Biol. 5, 17 (2007). doi: 10.1186/1741-7007-5-17

44. Y. Zhang "Template-based modeling and free modeling by I-TASSER in CASP7", Proteins 69, 108 (2007). doi: 10.1002/prot.21702

45. Y. Zhang "I-TASSER server for protein 3D structure prediction", BMC Bioinformatics 9, 40 (2008). doi: 10.1186/1471-2105-9-40

46. S. Hayer, M. Tohidast-Akrad, S. Haralambous, B. Jahn-Schmid, K. Skriner, S. Trembleau, H. Dumortier, S. Pinol-Roma, K. Redlich, G. Schett, S. Muller, G. Kollias, J.S. Smolen and G. Steiner, "Aberrant Expression of the Autoantigen Heterogeneous Nuclear Ribonucleoprotein-A2 (RA33) and Spontaneous Formation of Rheumatoid Arthritis-Associated Anti-RA33 Autoantibodies in TNF-alpha Transgenic Mice", J. Immunol. 175, 8327 (2005).

47. M.H. Hoffmann, J. Tuncel, K. Skriner, M. Tohidast-Akrad, B. Türk, S. Pinol-Roma, G. Serre, G. Schett, J.S. Smolen, R. Holmdahl and G. Steiner, "The rheumatoid arthritis-associated autoantigen hnRNP-A2 (RA33) is a major stimulator of autoimmunity in rats with pristane-induced arthritis", J. Immunol. 179, 7568 (2007).

48. B. Krol, R. Sanderman and T. Suurmeijer, "Social support, rheumatoid arthritis and quality of life: concept, measurement and research.", Patient Educ. Couns. 20, 101 (1993). doi: 10.1016/0738-3991(93)90125-G

8. Supplemental Material

Supplemental Table 1: Masses and sequence coverage of peptide fragments in bands (1-13) after BrCN degradation, SDS PAGE separation, and *in-gel* tryptic digest.

Band No. [a]	Exp. Mass (*m/z*)	Calc. Mass $[M+H]^+$; (Da)	Sequence Range
1	997.72	996.43	277–286 [b]
31 kDa	1085.71	1087.48	120-128
(1 – 286)	1377.49	1377.63	213-227
	1409.89	1410.69	173-184
	2780.28	2782.27	1-21
2	997.68	996.43	277–286 [b]
30 kDa	1377.64	1377.63	213-227
(13-286)			
3	1377.64	1377.63	213-227
29 kDa	1410.70	1410.69	173-184
(13-275)	1879.98	1879.96	113-128
	1927.02	1927.02	21-37
4	1013.42	1013.44	203-212
26 kDa	1087.75	1087.48	38-45
(13-192+X´)	1188.61	1188.64	137-146
	1377.75	1377.63	213-227
	1695.77	1695.76	153-167
	1410.69	1410.69	173-184
	1879.97	1879.96	113-128
	1927.01	1927.02	21-37
5	912.37	912.37	228-237
25 kDa	1013.42	1013.44	203-212
(72-314;	1121.54	1121.56	13-20
13-192+X´´)	1188.63	1188.64	137-146
	1191.66	1191.55	190–199 [c]
	1249.64	1249.66	13-21
	1338.67	1338.70	99-111
	1377.63	1377.62	213-227
	1410.69	1410.69	173-184
	1538.79	1538.77	173-185
	1695.77	1695.76	153-167
	1879.97	1879.97	113-128
	1927.01	1927.02	21-37
	2008.05	2008.06	112-128
	2433.26	2433.26	152-172
	2868.34	2867.39	22-45
	3039.44	3039.48	112-136
6	1188.44	1188.64	137-146
20 kDa	1410.36	1410.69	173-184
(13-192)	1695.98	1695.76	153-167
	1852.10	1851.87	152-167
	1879.48	1879.97	113-128
	2007.55	2008.06	112-128

Band No. [a]	Exp. Mass (*m/z*)	Calc. Mass $[M+H]^+$; (Da)	Sequence Range
7	1079.68	1079.56	79-88
18 kDa	1188.62	1188.64	137-146
(79-192+X´;	1410.69	1410.69	173-184
13-163)	1695.98	1695.76	153-167
	1852.11	1851.87	152-167
	1879.99	1879.97	113-128
	1927.03	1927.02	21-37
	2008.08	2008.06	112-128
8	1079.33	1079.56	79-88
15 kDa	1188.40	1188.64	137-146
(79-192+X´´)	1377.31	1377.63	213-227
	1410.36	1410.69	173-184
	1695.96	1695.76	153-167
	1852.09	1851.87	152-167
	1879.55	1879.97	113-128
	2007.64	2008.06	112-128
9	1079.33	1079.56	79-88
12 kDa	1087.22	1087.48	38-45
(79-192;	1188.34	1188.64	137-146
55-163	1256.64	1256.51	153–163 [d]
13-121)	1410.88	1410.69	173-184
	1412.81	1412.62	152-163 [d]
	1879.44	1879.97	113-128
	1926.49	1927.02	21-37
	2007.54	2008.06	112-128
	2185.63	2185.24	79-98
10	1087.42	1087.48	38-45
8 kDa	1927.02	1927.02	21-37
(1-71)			
11	1087.43	1087.48	38-45
6 kDa	1410.66	1410.69	173-184
	1927.02	1927.02	21-37
12	1927.01	1927.02	21-37
5 kDa			
13	1927.02	1927.02	21-37
3.5 kDa			

a) apparent molecular masses are given and estimated sequence ranges of predominant fragment(s) are indicated in parentheses

b) *C*-terminal homo-Ser lactone

c) conversion of internal Met residue to homo-Ser without cleavage

d) mild acid cleavage of Asp-Pro bond

III. Mass Spectrometric and Peptide Chip Epitope Analysis on the RA33 Autoantigen with Sera from Rheumatoid Arthritis Patients

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1. Abstract

As the potential of epitope chips for routine application in diagnostics relies on the careful selection of peptides, reliable epitope mapping results are of utmost interest to the medical community. Mass spectrometric epitope mapping in combination with peptide chip analysis showed that autoantibodies from patients who suffered from Rheumatoid Arthritis (RA) were directed against distinct surface structures on the full-length human autoantigen RA33 as well as against partial sequences. Using the combined mass spectrometric epitope extraction and peptide chip analysis approach, four sequence motifs on RA33 emerged as immuno-positive, showing that epitopes were not randomly distributed on the entire RA33 amino acid sequence. A sequential epitope motif (^{245}GYGGG^{249}) was determined on the *C*-terminal part of RA33 which matched with the Western blot patient screening results using the full-length protein and, thus, was regarded as a disease-associated epitope. Other epitope motifs were found on *N*-terminal partial sequences (^{59}RSRGFGF^{65}, ^{111}KKLFVG^{116}) and again on the *C*-terminal part (^{266}NQQPSNYG^{273}) of RA33. As recognition of these latter three motifs was also recorded by peptide chip analysis using control samples which were negative in the Western blot screening, these latter motifs were regarded as "cryptic epitopes". Knowledge of disease-associated epitopes is crucial for improving the design of a customized epitope peptide chip for RA and mass spectrometric epitope mapping pivotally assisted with selecting the most informative peptide(s) to be used for future diagnostic purposes.

Keywords

Rheumatoid Arthritis; Patient sera, RA33 autoantigen; Epitope mapping; Mass spectrometry; MALDI ToF MS; Peptide Chip analysis; Autoimmune diagnostics.

Abbreviations

CHCA: α-cyano-4-hydroxy-cinnamic acid

DHB: 2,5-dihydroxy-benzoic acid

RA33: rheumatoid arthritis autoantigen 33

TBS: TRIS buffered saline

ACN: acetonitrile

BrCN: cyanogen bromide

2. Introduction

RA33 (hnRNP A2/B1) was determined to be a major autoantigen in RA patients (prevalence is ca. 35 %), but patients with systemic lupus erythematosus (SLE; 23 %) and mixed connective tissue disease (MCTD; 39 %) have been found to also possess autoantibodies against RA33 [1]. Recently, it was suggested that antiRA33 autoantibodies in patients may be directed against different epitopes and that the recognition pattern was associated with the disease [2]. Truncated recombinant RA33 protein derivatives and RA33-derived synthetic peptides were tested with patient sera for immuno-positive reactions and lead to the conclusion that RA patients´ autoantibodies against RA33 recognized an assembled, *i.e.* a conformational epitope that was placed on domain II [3]. The presence of (auto)antibodies against sequential epitopes on RA33 in RA patient sera has as yet not been addressed. By contrast, it was found that antiRA33 autoantibodies from SLE patients were predominantly directed against a sequential, *i.e.* a linear epitope of RA33 comprising aa155-175 [2]. Hence, characterizing the epitope recognition specificities of autoantibodies in addition to the determination of the presence of autoantibodies against full-length antigens in autoimmune patients´ sera is regarded to be extremely helpful to improve the diagnostic and even prognostic value of a screening method which may guide individual therapy and on which therapy-related decisions may be based on.

Mass spectrometric and other epitope mapping procedures have become of tremendous importance for both, the identification of epitopes on known autoantigens and the estimation of cross-reactivities of antibodies with different antigens due to related epitope structures [4, 5]. Mass spectrometry-based epitope identification methods involve epitope excision [5, 6] and epitope extraction procedures [7, 8 9-11]. In our case, the epitope and non-epitope peptide fractions were analyzed by immuno-analytical methods, such as Western blot, and the epitope-containing sequences were identified directly by mass spectrometry. Our approach made use of the epitope extraction procedure [4] with sera from patients that contained polyclonal (auto)antibodies in conjunction with analyzing immuno-response of patient sera with peptide chips.

3. Patients, materials and methods

3.1 Patient samples and screening for antiRA33 autoantibodies

Twenty-four serum samples from 22 patients collected from the Rheumatology Clinics Vogelsang, University of Magdeburg, Germany, were included in this study (Table 1). The study was approved by the Institutional Review Board and blood samples were taken after informed consent was given. All 24 serum samples were investigated for the presence of autoantibodies against RA33 by Western-blot analysis as described [4]. Serum samples are used without further purification as used in other clinical and immunological screening assays. Recombinant RA33 was expressed in *E. coli* at the Euroimmun AG, Luebeck, Germany.

3.2 BrCN degradation of recombinant RA33, fragment separations, and immuno-analytical determinations

The BrCN-derived fragments of RA33 [4] were loaded onto 4-20 % TRIS glycine gels and separated by SDS-PAGE. Western Blot analysis was performed as described [4] using serum samples from patients 5 and 11 as antiRA33-antibody containing agents. Protein containing bands were manually excised and gel plugs were subjected to *in-gel* digestion with trypsin [12]. MALDI-ToF MS measurements were performed using a Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with the SCOUT source and operated in positive ion reflector mode [13].

The BrCN-derived fragments of RA33 were separated using the HPLC system as described [4] on an UltraSep ES PEPTID 125 x 2 mm column. For dot blot analysis lyophilized and HPLC-separated fractions of BrCN-derived fragments of RA33 were re-dissolved and dot blotted in triplicate using the MINIFOLD blotting device (Schleicher & Schuell/Whatman, Dassel, Germany) together with intact RA33 as a positive control and a blank (water) as a negative control [4]. Sera samples from patients 5 and 11 where used as antiRA33-antibody containing agents.

The HPLC-separated and lyophilized BrCN-derived fragments of RA33 were re-dissolved in 80 % ACN/0.1 % TFA. For MALDI ToF mass spectrometry in linear (matrix: saturated ferulic acid solution suspended in aqueous ACN/0.1 % TFA 33/67 v/v) as well as in reflector mode (saturated CHCA solution suspended in aqueous 35 % ACN/0.1 % TFA) sample (0.5 µl) and matrix solutions (0.5 µl) were added onto a stainless steel target and left to crystallize on air.

3.3 Peptide Chip Analysis

Replitope™ high density microarrays included peptides from RA33 autoantigen isoforms (heterogeneous nuclear ribonucleoprotein hnRNPA2/B1 RefSeq Accessions NP_002128 and NP_112533 (P22626)) as well as from hnRNPA1 (RefSeq NP_002127, NP_112420 (P09651)) and hnRNPAB (RefSeq NP_112556, NP_004490 (Q99729)) [4, 14]. Here, the chips were used to characterize the epitope binding profiles of sera against RA33 from patients 1, 5, 11, 13, and 24. To each patient sample set a matching "buffer-control" was recorded, *i.e.* buffer instead of patient serum was incubated on the chips. Strong signals in the buffer-control originated from secondary antibody binding and the respective peptides were excluded from the analysis. As background weak signals were regarded. Sample signal intensity was divided by background signal intensity for each peptide in order to classify response of antibodies (0, X, XX, XXX) to the respective motifs.

4. Results

4.1 Patient Screening

Twenty four serum samples from 22 patients were included in the study. All patients, except patient 3, were suffering from Rheumatoid Arthritis (RA) as was diagnosed by clinical means (*e.g.* disease activity score (DAS) and X-ray scan) and was substantiated by laboratory examinations (*e.g.* C-reactive protein (CRP)). From these samples we identified those RA patients that carried autoantibodies against RA33 by Western blot analysis using recombinantly produced RA33 (Table 1). The results showed that three from the 24 tested patient samples contained antiRA33 autoantibodies. Serum samples of the patients that were tested positive by Western blot screening (patients 1, 5, and 11) were subjected to further analyses (see below).

Table 1: Clinical and laboratory data of blood samples and results of Western blot analysis of RA patients.

Sample No.	Gender	Age [a]	ESR [mm/h]	DAS 28	CCP (GA) [U/ml]	CRP [mg/l]	Anti-RA33 Western blot
1	female	48 / 47	55	4.8	2096	156.2	**pos.** j)
2	female	42 / 37	14	2.9	< 25	27.0	neg.
3	male f)	45 / n.d. g)	11	n.d. g)	< 25	< 5	neg.
4	female	53 / 48	26	6.0	1689	< 5	neg.
5	male	71 / 62	27	n.d. g)	28	11.3	**pos.** j)
6	female	64 / 59	52	5.6	2238	54.8	neg.
7	female	49 / n.d. g)	20	n.d. g)	45	n.d. g)	neg.
8	female	67 / 59	54	5.1	2036	55.0	neg.
9	female	62 / 60	30	4.1	< 25	6.1	neg.
10	female	45 / 32	n.d. g)	6.0	141	8.8	neg.
11	female	37 / 26	65	7.2	403	107.0	**pos.** j)
12	female	61 / 50	6	4.4	< 25	< 5	neg.
13	female	49 / 28	34	6.3	> 3200	< 5	neg. j)
14	female	38 / 25	10	3.1	1307	< 5	neg.
15	female	68 / 64	64	5.1	2318	91.0	neg.
16	female	50 / 44	110	6.4	186	84.0	neg.
17	female	51 / 35	55	6.2	< 25	94.0	neg.
18	male	30 / 29	63	6.8	< 25	112.0	neg.
19	male	38 / 37	37	5.1	< 25	< 5	neg.
20	female	41 / 13	50	6.5	< 25	50.0	neg.
21	female h)	61 / 54	26	6.3	< 25	84.0	neg.
22	female h)	62 / 54	56	5.9	< 25	81.0	neg.
23	female i)	42 / 33	n.d. g)	n.d. g)	< 25	7.9	neg.
24	female i)	43 / 33	24	7.4	< 25	7.9	neg. j)

a) age of patient at time point of sample collection/at disease onset

b) ESR: erythrocyte sedimentation rate

c) DAS: disease activity score

d) CCP: anti-“cyclic citrullinated peptide”-antibody conc.; lower threshold: 25

e) CRP: C-reactive protein; lower threshold: 5

f) Psoriasis arthritis

g) n.d.: not documented

h) same patient,different time point of sample collection

i) same patient, different time point of sample collection

j) sample used for peptide chip analysis

In order to identify sequence regions on recombinant RA33 that contained epitopes against which the autoantibodies of the patients´ samples were directed, we generated RA33 fragments by BrCN degradation of the full-length protein and subjected these to epitope mapping experiments.

4.2 Mass spectrometric epitope mapping

4.2.1 SDS-PAGE, mass spectrometric peptide mapping, and Western blot

The BrCN-derived fragments of RA33 were first separated by SDS-PAGE. Colloidal Coomassie blue staining revealed that the RA33 protein was cleaved completely and 13 protein bands were generated which migrated to distinguishable positions in the gel with lower apparent molecular masses than that of the starting material (Figure 1A).

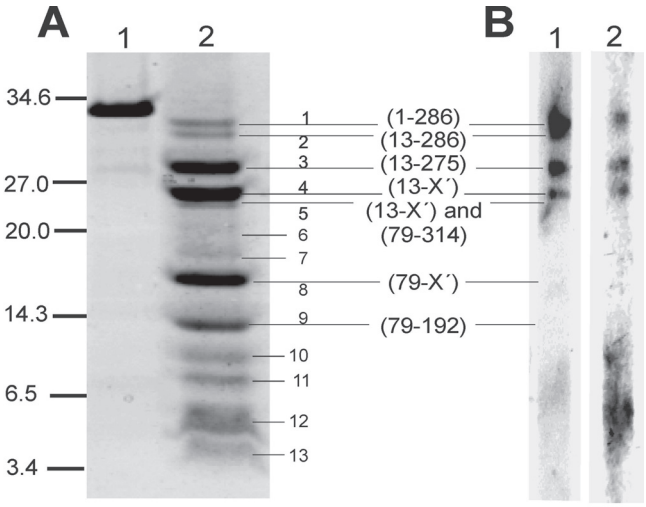

Figure 1: SDS PAGE and Western Blot analysis of BrCN-derived fragments of recombinant RA33. The SDS-PAGE image is aligned to the matching Western Blot image. **A**: 1D SDS PAGE. Lane 1: Full-length RA33. Lane 2: Bands of BrCN-derived fragments of RA33. Molecular mass marker positions are indicated on the left (numbers refer to apparent masses in kDa). Bands of BrCN-derived fragments are numbered beginning from the top. Major sequence ranges of recombinant RA33 that are displayed in the gel bands according to mass spectrometric peptide mapping analysis. **B**: Western Blot analysis using patient sera. Lane 1: Immuno-positive bands due to the reaction of the BrCN-derived fragments with serum from patient 5. Lane 2: Immuno-positive bands with serum from patient 11. Numbering of immuno-positive bands is printed in bold letters.

Mass spectrometric analysis of *in-gel* tryptic digested peptides from each band lead to the assignment of the sequence ranges that were present in the respective fragments.

A typical example is given for the analysis of the BrCN-derived fragments in band 5 (see below). In the MALDI ToF mass spectrum of the tryptic peptides from the RA33 fragment in band 5 (*cf*. Figure 1A) a peptide ion signal at *m/z* 1121.54 was assigned to the peptide ranging from aa13-20 of the full-length

RA33 sequence (Figure 2). Similarly, a peptide ion signal at *m/z* 1249.64 was assigned to peptide aa13-21. Both peptides can be explained by a BrCN cleavage at methionine residue 12 (Met12) at the N-terminus and a tryptic cleavage at the C-terminus at arginine residue 20 (Arg20) and lysine residue 21 (Lys21), respectively. Both peptides marked the most *N*-terminally located part of the RA33 fragment in this band. The molecular mass of this RA33 fragment in band 5 was estimated to ca. 25 kDa according to its migration behavior in SDS-PAGE. Accordingly, the *C*-terminal end of this fragment was to be located above the methionine residue at position 192 (Met192), which marked a potential BrCN cleavage site, but below Met275, the next more *C*-terminally positioned cleavage site for BrCN in the sequence. It is well known that during BrCN cleavage non-specific degradation may occur, yielding in additional fragments, *e.g.* by so-called "mild-acid" cleavage [15]. It was shown independently that the recombinant RA33 sample contained truncated protein side products [4] that may be responsible for the BrCN fragments in band 5 as well. As the precise *C*-terminal end of this fragment has not been determined so far, the assignment of the partial sequence of this fragment is given as aa13-X´, where the X´ marks a *C*-terminally located amino acid position beyond the residue Met192. Sequence assignments of some of the tryptic peptides were confirmed by MS/MS fragmentation (*cf.* Figure 2).

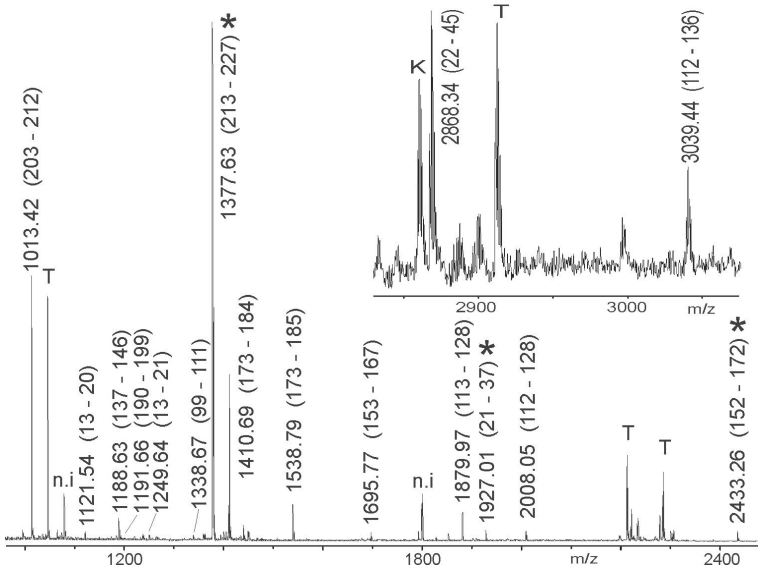

Figure 2: MALDI ToF MS peptide mapping analysis after in-gel trypsin digest of BrCN-derived protein fragments from immuno-positive band 5 (*cf.* Fig. 1). Peptide ion signals are labeled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses. T: ion signals due to trypsin auto-proteolysis; K: ion signals assigned to keratin; n.i.: not identified ion signals; *: assignment confirmed by MS/MS peptide sequencing. Matrix: CHCA.

Additionally, as the tryptic peptide mapping result showed that there are peptides which were matching with sequence parts positioned more *C*-terminal than the Met192 residue it was suggested that there might be a mixture of peptides co-migrating to the location of band 5 in the gel. The peptide ion signal at *m/z* 1377.63 was assigned to belong to the partial sequence aa213-227. From all possibilities to generate an RA33 fragment with ca. 25 kDa in size *via* BrCN cleavage, the best matching partial sequence was assumed to be fragment aa79-314 where BrCN had cleaved at Met78. Mass spectrometric fragmentation of the peptide with ion signal at *m/z* 1377.63 (aa213-227) resulted in a series of Y" ions and B-NH_3 ions that confirmed the partial sequence "GGNFGPGPGSNFR" (Figure 3). Hence, the MS/MS sequencing results substantiated the assumption that at least two co-migration BrCN-derived RA33 fragments were present in band 5.

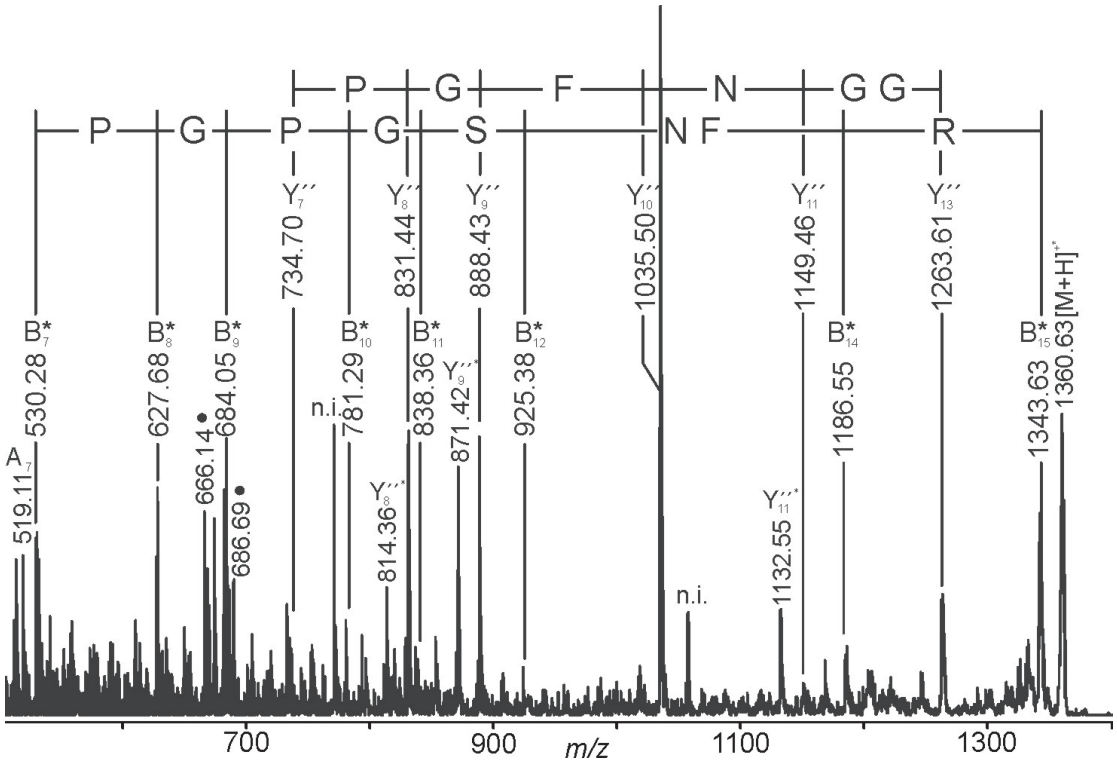

Figure 3: MALDI-QIT-ToF MS/MS spectrum of the ion signal at *m/z* 1377.63 (*cf.* Fig. 2). The determined partial amino acid sequence is depicted and was assigned to an RA33 peptide comprising amino acids 213-227. The mass spectrometric fragment ions from the Y"-type ion series and the B-NH_3-type ion series are indicated. The asterisk indicates loss of ammonia. A dot symbol indicates an internal fragment. DHB was used as matrix.

Western blot analysis with sera from patients 5 and 11, respectively, showed that the first five BrCN-derived bands stained positive, indicating that epitope(s)

were present in these RA33 fragments (Figure 1B). The staining pattern for the two tested serum samples was almost identical. Samples from patient 1 were not included in this experiment as there was too little sample amount left. As the strongly Coomassie stained, immuno-negative band 8 was assigned to contain the partial sequence aa79-X´, it was reasoned that the epitope regions for the autoantibodies in the tested patient sera were located either *N*-terminally, *i.e.* within the partial sequence aa13-78, or *C*-terminal to the (yet undefined) X´ position, aaX´-314, of the RA33 sequence (for summarized visualization of the results see Figure 6).

4.2.2 HPLC, mass spectrometric determination of BrCN fragments, and dot blot

In order to verify the results from SDS-PAGE and Western blot experiments we performed independent HPLC separations of the BrCN-derived RA33 fragments and tested the obtained HPLC fractions for their immuno-analytical behavior using dot-blot assays.

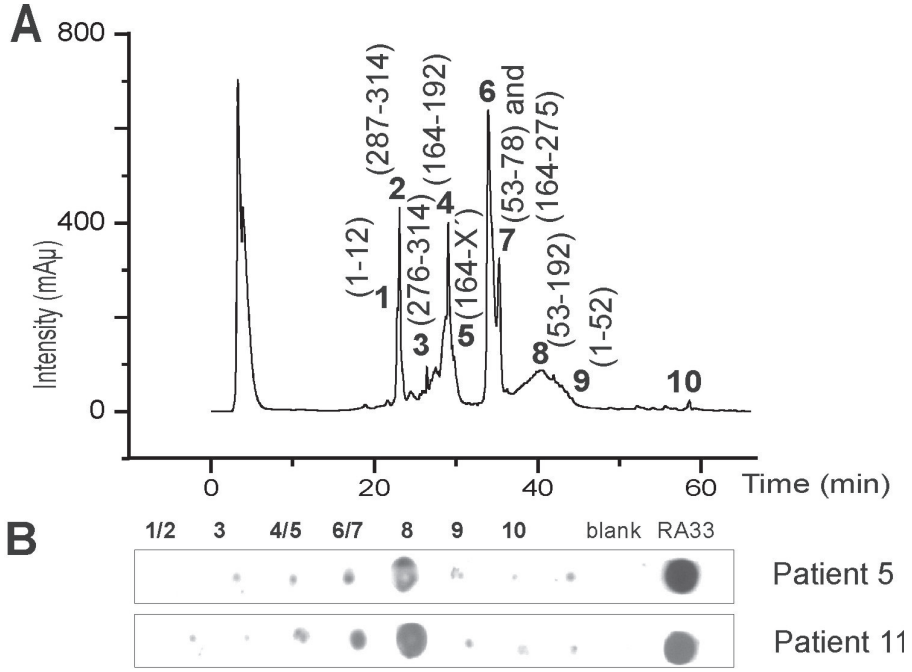

Figure 4: HPLC and dot blot analysis of BrCN-derived fragments of recombinant RA33. A: HPLC chromatogram of protein fragments after BrCN degradation. Numbers indicate major fractions that were collected and subjected to mass spectrometric and to dot blot analysis, respectively. Detection wavelength: 280 nm. Partial sequences of major BrCN-derived fragments are indicated in parentheses. B: Dot blot analysis of the HPLC fractions and a blank (negative control) as well as the full-length RA33 (positive control). Poorly separated fractions were pooled. Upper panel: sample from patient 5. Lower panel: patient 11.

Ten HPLC fractions were collected and subjected to mass spectrometric analysis [4]. In all cases the obtained spectra enabled to assign partial sequences of RA33 fragments to the respective fractions (Figure 4A).

As an example, mass spectrometric analysis of fraction 7 showed the presence of peptide ion signals in the low mass range that were assigned to the *N*-terminal partial sequence of RA33 around aa53-78 (Figure 5). All observed fragments were derived from BrCN cleavage where *C*-terminal methionine residues were turned into homoserine lactone and homoserine residues, respectively. In addition, an ion signal that was detected in the high mass range at *m/z* 8212 was assigned to a *C*-terminal BrCN-derived fragment of RA33 comprising the partial sequence aa193-275. The peptide assignment is consistent with a homoserine lactone residue at the C-terminus and a pyro-glutamic acid residue at the N-terminus. Conversion of glutamines or glutamic acids into pyro-glutamic acids during BrCN cleavage has been described [16].

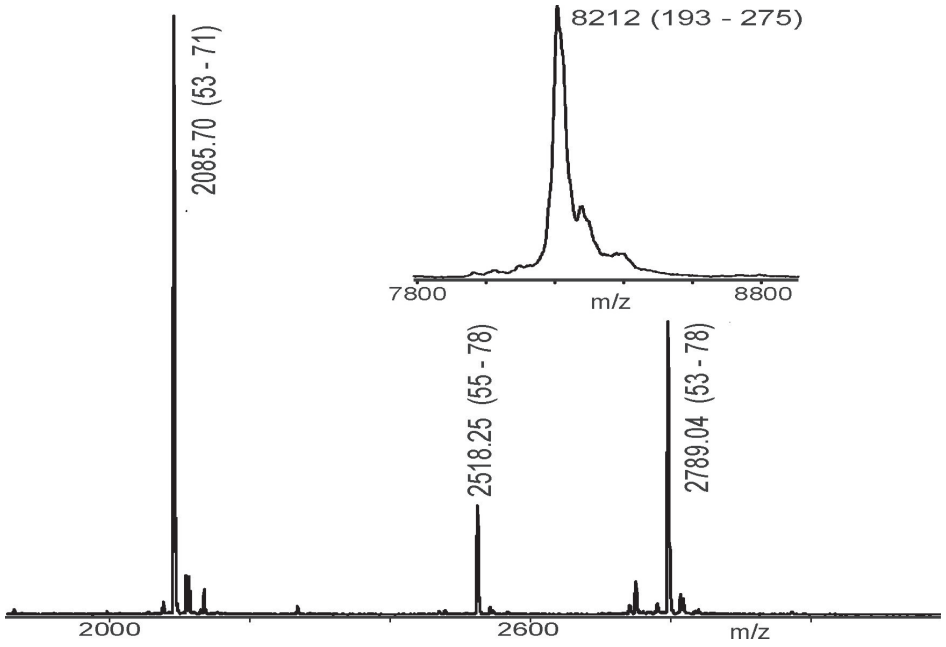

Figure 5: MALDI ToF MS analysis of a protein fragment from immuno-positive HPLC fraction 7 (*cf.* Figure 3). The spectrum was recorded in reflector mode (*m/z*: 800–3500). The linear mode spectrum from *m/z* 7800–8800 is shown in the insert. Matrix: Ferulic acid. Peptide ion signals are labeled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses.

Dot blot analysis with separated HPLC fractions with autoantibodies from patient sera showed that peptides in fraction 8 were giving an intensely stained dot. A weaker signal was obtained from peptides in pooled fractions 6/7 when compared to the positive control (full length recombinant RA33). The signal

intensities in the other fractions only reached background levels (Figure 4B).

Taking all immuno-staining information from the HPLC fractions together, we inferred from these experiments that the epitopes from the autoantigens in the tested patient sera (patients 5 and 11) were located in an *N*-terminal region comprising aa53-78 and in a *C*-terminal region that was located between the yet undefined position X´ and Met275 (aaX´-275). This result is consistent with that obtained by SDS-PAGE combined with Western blot analysis of the RA33 fragments but further confined the epitope containing regions (*cf*. Figure 6; grey bars).

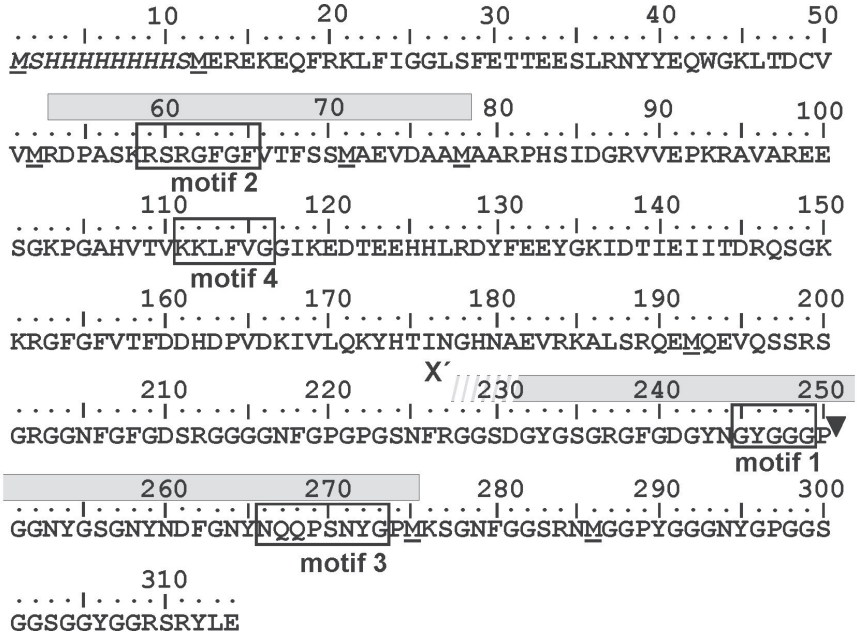

Figure 6: Amino acid sequence of RA33 in single letter code. The protein sequence resembles the A2 isoform of RA33 (Acc. No. P22626) with a His tag at the N-terminus (printed in italic letters). The triangle maps the position, where the A2 isoform of RA33 bears an additional sequence stretch of 40 amino acids that is lacking in the recombinant RA33. Methionine residues are underlined. The shaded bars indicate the areas which were addressed as epitope carrying regions by mass spectrometric epitope mapping. X´ marks a potential location of the yet undefined *N*-terminal border of the *C*-terminal epitope region. The boxes display the epitope motifs that are recognized by the antibodies from the patient sera using peptide chip analysis (*cf*. Table 2).

4.2.3 Peptide chip epitope analysis

Additionally, peptide chips were applied for studying interactions of (auto)antibodies from patient sera. Here, RA33 partial sequences were represented by 15-meric synthetic peptides. The chips were designed such that the full-length RA33 sequence was displayed by 59 peptides with a sequence overlap of 9 amino acids, each [4]. All peptides were present in triplicate, allowing to simultaneously collect redundant information as technical replicates. Due to the sequence overlap it was possible to acquire redundant information with respect to describing epitope sequence motifs. Serum samples from three RA-positive patients (1, 5, and 11) were analyzed (Table 2) and from patient 11 we analyzed three independent serum samples that were collected at different time points (samples 11_1, 11_2, and 11_3). These latter samples may be regarded as biological replicates. The peptide chip results with these biological replicates showed high consistency, emphasizing the robustness of the method. The best scoring peptides were used to search over-represented motifs in the most significant peptides applying the PRATT Pattern matching tool [17]. Four motif groups emerged (*cf.* Figure 6), summarized as epitope motifs 1 (^{245}GYGGG^{249}; best fitness: 19.9), 2 (^{59}RSRGFGF^{65}; 24.1), 3 (^{266}NQQPSNYG^{273}; 34.6), and 4 (^{111}KKLFVG^{116}; 19.0).

Table 2: Peptide chip analysis of RA33 autoantigen peptides probed with serum samples from RA patients [a].

patient	motif 1 [b] ^{245}GYGGG^{249}	motif 2 [c] ^{59}RSRGFGF^{65}	motif 3 [d] ^{266}NQQPSNYG^{273}	motif 4 [e] ^{111}KKLFVG^{116}	RA33 WB
1 [g]	X	XXX	XXX	0	pos.
5 [g]	XX	XXX	XX	0	pos.
11_1 [g]	XXX	X	XX	XX	pos.
11_2 [g]	XXX	0	X	XX	pos.
11_3 [g]	XXX	X	X	X	pos.
13	0	XX	X	XXX	neg.
24	0	XX	XX	XX	neg.

a) 0 = signal less intense than 3x that of control
X = signal at least 3x more intense than that of control
XX = signal at least 10x more intense than that of control
XXX = signal at least 20x more intense than that of control
b) motif is present in 6 of the 19 best scoring peptides out of 5 chips
c) motif is present in 5 of the 19 best scoring peptides out of 5 chips
d) motif is present in 3 of the 19 best scoring peptides out of 5 chips
e) motif is present in 3 of the 19 best scoring peptides out of 5 chips
f) full length RA33
g) peptide chip was used to select best scoring peptides

Autoantibodies from patient 11 showed strong signals with epitope motif 1 and less intense signals for the epitope motifs 2, 3, and 4. Hence, it can be concluded that the amino acid sequence ^{245}GYGGG^{249} represents a dominant sequential RA33 epitope in this patient. The same epitope motif was also recognized by autoantibodies from patients 1 and 5 with somewhat lesser intensities on the chips. Epitope motifs 2 and 3 displayed stronger signals with the latter two patient samples whereas there was no detectable response with epitope motif 4 (Table 2).

It is important to note that from the entire RA33 amino acid sequence only four short peptide motifs emerged as epitopes by peptide chip analysis. This result indicated (i) that epitopes were not randomly distributed on the protein sequence, and (ii) that common epitopes were shared between patients. However, on top of the common epitope recognition a "patient specific" pattern of epitope binding could be observed by the peptide chip analyses as well.

We also studied the peptide chip responses with (auto)antibodies from RA33-negative RA patients as controls, *i.e.* patients whose sera did not give positive responses in Western blot analyses using the full-length RA33 (*cf.* Table 1). Interestingly, epitope motifs 2-4 yielded in response signals due to binding of antibodies from these sera to the respective peptides. This indicated that these epitope motifs might be regarded as so-called "cryptic" epitopes, as the respective sequence ranges were not recognized by the patients´ (auto)antibodies when displaying the full-length protein. In summary, only motif 1 was recognized by (auto)antibodies from RA33-positive patients (*cf.* Table 1) and, hence, remained the only epitope motif that correlated with both, the mass spectrometric epitope mapping results and the Western blot results obtained with the full-length recombinant RA33.

In order to estimate surface accessibility of the discussed epitope motifs on the full-length RA33 protein we mapped the epitope sequences on a 3D model of recombinant RA33 (Figure 7).

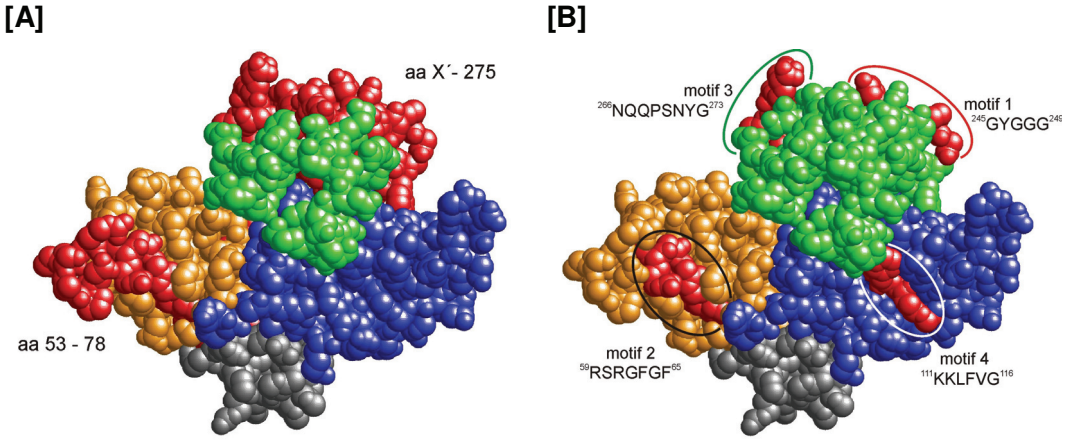

Figure 7: Structural representations of recombinant RA33. The three-dimensional structure representation has been modeled using the "I-TASSER" algorithm (http://zhang.bioinformatics.ku.edu/I-TASSER). The molecular surface is indicated with van-der-Waals spheres. The His tag region is displayed in black, domain I in yellow, domain II in blue, and domain III in green. **A**: The epitope-containing regions as determined by mass spectrometry are shown in red. **B**: The four epitope motifs as derived form the peptide chip analysis are displayed in red and are circled. Sequence ranges of the motifs are given.

The model made use of a partial tertiary structure that encompasses the two *N*-terminal domains of a related protein (protein data base entry: 1HA1). The third domain (*C*-terminal domain) was modeled on top of this structure using the I-TASSER program [18, 19]. Interestingly, epitope motifs 1 and 3 appeared well accessible on the surface of domain III (co-localizing with the mass spectrometrically defined epitope-containing region X´-275). However, as no crystal structure of domain III is available, the actual surface areas of the epitope motifs 1 and 3 cannot be determined. Motifs 2 and 4 seemed fairly buried in domain I and in domain II, respectively (Figure 7). It should be noted that the mass spectrometrically derived epitope-containing partial sequence 53-78 encompassed motif 2 on domain I but not motif 4 on domain II.

5. Discussion

The aim of our studies was to emerge a disease-associated epitope pattern that ultimately shall enable to stratify patients for selecting the best available therapy for each individual. Mapping of epitope regions on (auto)antigens using patient sera that contained polyclonal (auto)antibodies should result in the assignment of more than one epitope on a given (auto)antigen, when these polyclonal (auto)antibodies are directed against different surface structures of the

(auto)antigen under study. Hence, detection of four major epitope motifs on RA33 against which (auto)antibodies from patient sera are directed is, at first hand, not surprising.

The existence of epitopes in the *C*-terminal region of RA33 against which autoantibodies from RA patients were directed has been mentioned [1]. However, distinct epitopes in that region have not yet been defined. It is of importance to note that the *C*-terminal regions of RA33 were mostly neglected in earlier epitope identification analyses [2, 3, 20, 21]. Instead, focus had been laid onto *N*-terminal epitope regions.

Our combined mass spectrometric epitope extraction and peptide chip analysis approach specifically addresses sequential epitopes (named linear or continuous epitopes in many literature references). The simultaneous presence of (auto)antibodies directed against structurally assembled epitopes (also termed conformational or discontinuous epitopes) on an (auto)antigen is not ruled out.

A general issue to be addressed in epitope mapping experiments with truncated partial proteins or peptides is the recognition of partial structures by the (auto)antibodies that are displayed only when parts of the full-length molecule are absent. In these cases the recognition of so-called "cryptic epitopes" has to be considered [4]. Cryptic epitope responses should not match with analysis results from assays (*e.g.* Western blot) in which the full-length protein is used. As a consequence, presenting surface-bound peptides to patient sera (bottom-up approach) should be accompanied by epitope determination strategies that start out with the full-length antigen (top-down approach). Comparison of the results from the two independent approaches is recommended for interpreting epitope mapping data.

Epitope extraction describes the proteolytic digestion of the free antigen and presentation of the mixture of peptide fragments to the antibody under investigation, followed by isolation of the antibody-peptide complex from which the epitope-containing peptide is liberated prior to analysis [4-8]. The simultaneous preparation of the epitope peptide bound antibody complex directly on the MALDI target has also been demonstrated by using monoclonal antibodies [9, 10].

It should be noted that three of the four peptide chip-derived epitope motifs fell within the regions that were assigned as epitope carrying partial sequences from the mass spectrometric epitope mapping analysis (*cf.* Figure 6). This showed that BrCN-derived fragments of RA33 also displayed "cryptic" epitopes

in addition to so-called "disease-associated" sequential epitopes. The corroborated information seems crucial when aiming at epitope-based screening applications for diagnostics.

For patient screening purposes the application of peptide chips seems more robust and less error prone than the use of full-length antigen arrays [14]. The use of epitope chips eliminates all potential cross-reactivities that are always of concern with full-length autoantigens that may give rise to false positive reactions. Therefore, the goal of this study was to identify such epitope peptides that are disease-associated. Whether epitope chips shall assist or even replace antigen-based diagnostic assays in the future relies on the experiences that shall be made with respect to specificity and sensitivity of these assays, *i.e.* false positive and false negative rates. The ultimate performance of a peptide chip in this respect certainly relies on the careful selection of peptides, catapulting reliable epitope mapping results into the center of interest.

6. Acknowledgements

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7. References

1. G. Steiner, K. Skriner, W. Hassfeld and J.S. Smolen, "Clinical and immunological aspects of autoantibodies to RA33/hnRNP-A/B proteins-a link between RA, SLE and MCTD", Mol. Biol. Rep. 23, 167 (1996). doi: 10.1007/BF00351165

2. G. Schett, H. Dumortier, E. Hoefler, S. Muller and G. Steiner, "B cell epitopes of the heterogeneous nuclear ribonucleoprotein A2: identification of a new specific antibody marker for active lupus disease", Ann. Rheum. Dis. 68, 729 (2009). doi: 10.1136/ard.2007.087502

3. K. Skriner, W.H. Sommergruber, V. Tremmel, I. Fischer, A. Barta, J.S. Smolen and G. Steiner, "Anti-A2/RA33 Autoantibodies Are Directed to the RNA Binding Region of the A2 Protein of the Heterogeneous Nuclear Ribonucleoprotein Complex", J. Clin. Invest. 100, 127 (1997). doi: 10.1172/JCI119504

4. R.F. ElKased, C. Koy, T. Deierling, P. Lorenz, Z. Qian, Y. Li, H.-J. Thiesen and M.O. Glocker "Mass Spectrometric and Peptide Chip Epitope Mapping of Rheumatoid Arthritis Autoantigen RA33 ", Eur. J. Mass Spectrom. 15, 747 (2009).

5. M. Macht, A. Marquardt, S.-O. Deininger, E. Damoc, M. Kohlmann and M. Przybylski, ""Affinity-proteomics": direct protein identification from biological material using mass spectrometric epitope mapping", Anal. Bioanal. Chem. 378, 1102 (2004). doi: 10.1007/s00216-003-2159-8

6. E.O. Hochleitner, C. Borchers, C. Parker, R.J. Bienstock and K.B. Tomer, "Characterization of a discontinuous epitope of the human immunodeficiency virus (HIV) core protein p24 by epitope excision and differential chemical modification followed by mass spectrometric peptide mapping analysis", Protein Sci. 9, 487 (2000).

7. D. Suckau, J. Kohl, G. Karwath, K. Schneider, M. Casaretto, D. Bitter-Suermann and M. Przybylski, "Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping", Proc. Natl. Acad. Sci. 87, 9848 (1990).

8. L. Yu, S.J. Gaskell and J.L. Brookman, "Epitope mapping of monoclonal antibodies by mass spectrometry: identification of protein antigens in complex biological systems", J. Am. Soc. Mass Spectrom. 9, 208 (1998). doi: 10.1016/S1044-0305(97)00250-X

9. J.G. Kiselar and K.M. Downard, "Direct identification of protein epitopes by mass spectrometry without immobilization of antibody and isolation of antibody-peptide complexes.", Anal. Chem. 71, 1792 (1999). doi: 10.1021/ac9811120

10. B. Morrissey and K.M. Downard, "A proteomics approach to survey the antigenicity of the influenza virus by mass spectrometry", Proteomics 6, 2034 (2006). doi: 10.1002/pmic.200500642

11. Y. Zhao and B.T. Chait, "Protein Epitope Mapping by Mass Spectrometry", Anal. Chem. 66, 3723 (1994). doi: 0003-2700/94/0366-3723$04.05/0

12. J.C. Heitner, C. Koy, T. Reimer, M. Kreutzer, B. Gerber and M.O. Glocker, "Differentiation of HELLP Patients from Healthy Pregnant Women by Proteome Analysis", J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 840, 10 (2006). doi: 10.1016/j.jchromb.2006.06.002

13. M. Kienbaum, C. Koy, H.V. Montgomery, S. Drynda, P. Lorenz, H. Illges, K. Tanaka, J.

Kekow, R. Guthke, H.-J. Thiesen and M.O. Glocker "Mass Spectrometric Characterization of Apheresis Samples from Rheumatoid Arthritis Patients for the Improvement of Immunoadsorption Therapy-A Pilot Study", Proteomics Clin. Appl. 3, 797 (2009). doi: 10.1002/prca.200800232

14. P. Lorenz, M. Kreutzer, J. Zerweck, M. Schutkowski and H.-J. Thiesen, in: Methods in Molecular Biology Epitope Mapping Protocols; U. Reineke and M. Schutkowski, Eds., Humana Press, Vol. 524, pp 247 (2009).

15. M.O. Glocker, B. Arbogast, J. Schreurs and M.L. Deinzer, "Assignment of the inter- and intramolecular disulfide linkages in recombinant human macrophage colony stimulating factor using fast atom bombardment mass spectrometry", Biochemistry 32, 482 (1993). doi: 10.1021/bi00053a012

16. K. Strohmaier, R. Franze and K.-H. Adam, "Location and characterization of the antigenic portion of the FMDV immunizing protein", The Journal of General Virology 59, 295 (1982).

17. I. Jonassen, J.F. Collins and D.G. Higgins, "Finding flexible patterns in unaligned protein sequences", Protein Sci. 4, 1587 (1995). doi: 10.1002/pro.5560040817

18. S. Wu, J. Skolnick and Y. Zhang, "Ab initio modeling of small proteins by iterative TASSER simulations", BMC Biol. 5, 17 (2007). doi: 10.1186/1741-7007-5-17

19. Y. Zhang "I-TASSER server for protein 3D structure prediction", BMC Bioinformatics 9, 40 (2008). doi: 10.1186/1471-2105-9-40

20. H. Dumortier, F. Monneaux, B. Jahn-Schmid, J.-P. Briand, K. Skriner, P.L. Cohen, J.S. Smolen, G. Steiner and S. Muller, "B and T cell responses to the spliceosomal heterogeneous nuclear ribonucleoproteins A2 and B1 in normal and lupus mice", J. Immunol. 165, 2297 (2000).

21. S. Hayer, M. Tohidast-Akrad, S. Haralambous, B. Jahn-Schmid, K. Skriner, S. Trembleau, H. Dumortier, S. Pinol-Roma, K. Redlich, G. Schett, S. Muller, G. Kollias, J.S. Smolen and G. Steiner, "Aberrant Expression of the Autoantigen Heterogeneous Nuclear Ribonucleoprotein-A2 (RA33) and Spontaneous Formation of Rheumatoid Arthritis-Associated Anti-RA33 Autoantibodies in TNF-alpha Transgenic Mice", J. Immunol. 175, 8327 (2005).

IV. A Novel Mass Spectrometric Epitope Mapping Approach Without Immobilization of the Antibody

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1. Abstract

The development and application of a mass spectrometry-based epitope mapping procedure in solution and without immobilization of the antibody is described. Antigens were digested with proteases. Then size exclusion micro-column chromatography (SEC) was carried out prior to and upon exposure of the peptide mixtures to monoclonal antibodies. The epitope-containing peptide, affinity bound to the antibody and, thus, forming a stable complex eluted early as did all high-mass components, whereas all unbound low-mass peptides eluted late. Comparison of elution profiles in the presence and absence of the antibody showed a shift only for epitope-bearing peptides, enabling direct identification of the epitope. His-tag containing recombinant antigens Fibrillarin and RA33 were used in combination with an anti-His-tag monoclonal antibody in order to develop the method. Application of the method for the determination of an epitope on RA33 against which a monoclonal antibody was directed identified the epitope sequence (^{85}IDGRVVEPKRA^{95}) using MS/MS peptide sequencing. Advantages of this approach include low sample consumption, few handling steps, and short duration of analysis. With our method we are ultimately aiming at developing a screening procedure to identify major epitopes in patients that may be suitable in the future for stratification of patients, needed for personalized therapies.

Keywords

epitope mapping, epitope extraction, enzymatic proteolysis, size exclusion chromatography, matrix assisted laser desorption/ionization mass spectrometry, monoclonal antibody

Abbreviations

aa: amino acid, DHB: 2,5-dihydroxy benzoic acid, RA33: rheumatoid arthritis auto-antigen33, ACN: acetonitrile, TFA: trifluoroacetic acid, NH_4HCO_3: ammonium bicarbonate, TBS: tris buffered saline, BSA: bovine serum albumin

Short Title

In-solution epitope mapping

2. Introduction

Epitope mapping is the process of identification of the molecular determinants for antibody–antigen recognition on the antigen. Identification of the epitope is a key step in the characterization of monoclonal antibodies, especially those used in therapeutic strategies (Nelson et al., 2000). It has also been essential in the design of vaccines against toxins (Kazemi and Finkelstein, 1991; Logan et al., 1991) or against enzymes (Gonzalez et al., 1994; Saint-Remy, 1997). To obtain information on sequential and/or assembled epitopes, various approaches have been developed in conjunction with mass spectrometry (Suckau et al., 1990; Hager-Braun and Tomer, 2005). Fine epitope mapping could be achieved by this approach with microgram quantities of antibodies and in a short time compared to conventional mapping methods (Dhungana et al., 2009; Macht et al., 1996; Parker et al., 1996; Suckau et al., 1990). The epitope analysis can be performed by epitope excision (Juszczyk et al., 2009; Hager-Braun et al., 2006; Hochleitner et al., 2000; Suckau et al., 1990; Przybylski, 1994) or by epitope extraction (Tian et al., 2007; Bílková et al., 2005; Zhao et al., 1996; Zhao and Chait, 1994). In both cases the method is based on the fact that antibodies in general are very resistant to proteolytic digestion whereas the antigen has to be readily degradable.

In epitope excision studies, mass spectrometry was used to identify epitope peptides released from an immobilized antibody-antigen complex after limited proteolysis (Pimenova et al., 2009; Parker and Tomer, 2002; Suckau et al., 1990). Here, the antibody prevents either proteolysis (Jemmerson, 1996) or chemical modification (Burnens et al., 1987) of sites of the antigen that are situated in the antibody binding pocket. However, because of the steric hindrance between bound antibody and proteolytic enzyme, the determined region is generally larger than the protected epitope that is recognized by the antibody as binding site (Manca, 1991). An important feature of this approach is that, because non-denaturing conditions are used, the antigen retains its native conformation so that assembled (conformational) epitopes can be determined (Parker and Tomer, 2000).

In epitope extraction, a protease digested antigen peptide mixture is in most cases affinity-bound to an immobilized antibody. The epitope peptides are analyzed by MALDI-MS after washing off all unbound peptides and upon release of the epitope peptide from the immobilized antibody. Subsequently, sequential (linear) epitopes are identified directly by mass spectrometry (Bílková et al., 2005; Parker and Tomer, 2002).

In both approaches the peptides containing the epitopes are dissociated from the immobilized antibodies by elution with low pH buffer. The high accuracy and sensitivity of mass spectrometry helps in identifying the peptides that are regarded as epitopes. Furthermore, the resulting antibody-peptide complex must survive all procedures and washing steps prior to release of the epitope peptides. These mass spectrometry based methods in general require that the antibody be immobilized prior to reaction with antigen. Also critical to the success of this method is the choice of the proteolytic enzyme by which the antigen is being degraded and the already mentioned given stability of the antibody against proteolysis.

In contrast to the procedure described above, an alternative epitope extraction procedure without immobilization of the antibody was described where epitope peptides were identified from a direct comparison of the MALDI mass spectra of the peptide control mixture with that following interaction with antibody. Disappearance of peptide ion signals in the mass spectra from the antibody reaction mixture was interpreted as a result of antibody-binding to the epitope sequences within the protein (Kiselar and Downard, 1999).

Our study presents a simplified approach that maps the epitope on a given antigen also without immobilization of the antibody and without a separate step to dissociate the epitope peptide from the antibody-peptide complex prior to mass spectrometric analysis. The antigen-antibody complex is maintained in solution throughout all steps needed for separating epitope-containing peptide from all non-binding peptides affinity bound to the antibody using size-exclusion chromatography. As size exclusion chromatography is a method readily applied to the analysis of intact molecular weights of antibodies and antibody conjugates (Brady et al., 2008; Lazar et al., 2005) the overall handling procedure is simple and straight forward. In contrast to previous reports, dissociation of the epitope peptides from the antibody occurs simultaneously to the preparation of the peptides for MALDI-MS analysis with acidic matrices. Our novel epitope mapping approach was developed using a model system consisting of a commercially available monoclonal antibody directed towards the His-tag sequence present in recombinant Fibrillarin and RA33 proteins. Our method was further applied successfully to a known system composed of a monoclonal antibody directed towards its sequential epitope on RA33 (El-Kased et al., 2009).

3. Materials and methods

3.1 Proteins and antibodies

Recombinant Fibrillarin protein derivative comprising amino acid range 76-321 of the full-length protein was a gift from the Institute of Immunology, University of Rostock, Germany. The protein was obtained in solution consisting of 50 mM sodium phosphate, pH 8, 0.3 M sodium chloride, 0.05% Tween 20 and 8 M urea, with a protein concentration of 1.21 mg ml^{-1}. Microcon® centrifugal filter devices YM-10 [Millipore, USA] were used to desalt and concentrate the protein solution according to the manufacturer`s protocol. 300 µl of protein solution were rebuffered and concentrated to a protein concentration of 2.25 mg ml^{-1} in ammonium bicarbonate buffer, pH 8.5. The solution was then aliquoted (30 µl each) and stored at -20 ºC. Recombinant RA33 protein was purchased from Euroimmun AG, Luebeck, Germany. This autoantigen was shipped in solution consisting of 50 mM sodium phosphate, pH 7.4; 8 M urea; and 1000 mM sodium chloride, with a protein concentration of 2.42 mg ml^{-1}. 100 µl protein solution were quantitatively precipitated using 400 µl methanol/100 µl chloroform solution and resolubilised in 1000 µl 3 mM TRIS/HCl, pH 8.5, then aliquoted (30 µl each) and stored at -20 ºC. Penta-His antibody was purchased lyophilized from QIAGEN® GmbH [Hilden, Germany]. The lyophilized antibody (100 µg) was dissolved in 100 µl distilled water resulting in a protein concentration of 1 mg ml^{-1}. Anti-Histidine tag monoclonal antibody was purchased from AbD Serotec [Düsseldorf, Germany] in a phosphate buffered saline, pH 7.4, with a protein concentration of 1 mg ml^{-1}. Anti-RA33 monoclonal antibody (mouse anti-hnRNP-A2/B1) was purchased from Sigma [Munich, Germany]. The antibody was in solution consisting of 0.01 M phosphate buffered saline, pH 7.4, and 15 mM sodium azide, with a protein concentration of 1.5 mg ml^{-1}.

3.2 Proteolytic digests

Recombinant protein aliquots, dissolved in 30 µl of buffer (see above), were reduced with 3 µl 0.1 M freshly prepared dithiothreitol solution (15 mg DTT powder dissolved in 1 ml distilled water) at 56 ºC for 30 min. Subsequently, freshly prepared iodoacetamide solution (6 µl; 18.5 mg IAA powder dissolved in 1 ml distilled water) was added at room temperature and incubated in the dark for 30 min in order to alkylate sulfhydryl groups. For in-solution tryptic digest, an enzyme solution of 1 mg ml^{-1} in 3 mM TRIS/HCl, pH 8.5 was used [Promega, Madison, WI, USA]. 1.35 µl and 1.45 µl, respectively, enzyme solution were added. Enzyme:substrate ratio was 1:50. In both experiments digestion was

incubated overnight at room temperature. For in-solution thermolytic digest; an enzyme solution of 1 mg ml^{-1} in sodium acetate buffer, pH 8.5 was used [Sigma, Munich, Germany]. 1.45 µl enzyme solution were added to 30 µl RA33 solution with enzyme:substrate ratio of 1:50. The mixture was incubated overnight at room temperature.

3.3 Incubation of proteolytic peptide mixtures derived from antigens with antibody prior to size exclusion

66.7 pmol anti-His antibody were incubated overnight at room temperature in two separate experiments with 17.5 pmol tryptic digest peptide mixture derived from Fibrillarin and 17.5 pmol tryptic digest peptide mixture derived from RA33, respectively. 100 pmol monoclonal antibody were incubated overnight at room temperature with 35 pmol thermolytic digest peptide mixture derived from RA33.

3.4 Size exclusion chromatography of proteolytic peptides in the presence of the antibody

1 ml pipette tip (1000 µl blue tip, Greiner Bio-one GmbH, Frickenhausen, Germany) was used as a microcolumn. First, a small filter paper [MoBiTec, Goettingen, Germany] was inserted manually to act as a frit. Second, 250 µl of the gel slurry [Superdex 30; GE Healthcare] were added after vigorous shaking. After precipitation of the gel, the supernatant was pushed through and discarded. Such prepared tips were first washed with 100 µl water and then equilibrated with 100 µl of the elution buffer. 50 mM ammonium bicarbonate, pH 8.5 [Fluka, Germany] and 10% ACN/0.1% TFA, pH 2.2, respectively, were used as elution buffers. All solutions were loaded onto the top of the microcolumn using a pipette and the liquid was pressed through the microcolumn using an air-filled plastic syringe. For peptide elution, 10 µl of sample were loaded and allowed to enter the column bed. Next, 10 µl of elution buffer were loaded and gently pushed through the column, generating fraction 1. This procedure was repeated with addition of 10 µl of buffer each, until 10 fractions were collected in total.

3.5 SDS PAGE analysis

5 µl of sample were mixed with 5 µl two-fold tricine SDS sample buffer containing 100 mM Tris-HCl, pH 6.8, 0.3 % SDS, 24 % glycerol (w/v), 8 % SDS (w/v), 5 % 2-mercaptoethanol (v/v) and 0.02 % Bromophenol Blue (w/v). This sample solution (10 µl) was loaded onto a 16 % tricine gel, 1.00 mm x 10 well [Invitrogen, Karlsruhe, Germany]. The Broad Range Marker [New England BioLabs, Frankfurt/Main, Germany] as well as the Ultralow Range Marker

[Sigma, Munich, Germany] were used for determining apparent molecular masses of the protein bands. Gels were run in tricine SDS running buffer (1M Tris, 1M tricine, 1% SDS) (Schägger et al., 1988), using the Xcell Surelock™ MiniCell electrophoresis chamber [Invitrogen, Karlsruhe, Germany] at a constant voltage of 125 V for 60 min. Gels were fixed with a 50 % ethanol/10% acetic acid/water solution and stained with colloidal Coomassie Brillant Blue G-250 (Neuhoff et al., 1988).

3.6 Western blot and Dot blot analysis

For Western blot analysis; 1D SDS PAGE was performed. Protein bands were blotted onto a PVDF membrane [Immobilon, Millipore, Schwalbach, Germany] by semi-dry blotting for 60 min with a current of 1.2 mA/cm^2. The membrane was cut into strips. After blocking the strips with 2 ml blocking buffer (TBS 5 % non-fat dry milk powder, 1 % BSA 0.02 % thimerosal) for 2 h, one RA33 protein strip was incubated with the monoclonal antibody (1:1900) over night at 4 °C. A second RA33 protein strip and a Fibrillarin protein strip were incubated with the anti-His antibody (1:1900) overnight at 4°C. Washing (three times, in TBS, 0.05 % TWEEN 20 (v/v), 0.1 % BSA (w/v), 0.02 % thimerosal (w/v)) was followed by secondary antibody reaction (biotin-GAH IgG + IgM anti-mouse, 1:20,000, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA/Dianova) for 1 h at room temperature. Another washing step and reaction with streptavidin peroxidase (1:10,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA/Dianova) was carried out for 1 h at room temperature. Detection was performed using the SuperSignal West Pico Chemiluminiscent substrate [Pierce/ThermoFisher Scientific, Bonn, Germany]. Images were electronically processed to enhance band patterns.

For dot-blot analysis, fractions were collected after size exclusion chromatography of a mixture of the monoclonal antibody that was incubated with thermolysin. The MINIFOLD blotting device [Schleicher & Schuell/ Whatman, Dassel, Germany] was used and an antibody sample prior to elution was used as a positive control. After dot blotting, the membrane was taken out of the device and blocking as well as antibody incubation was performed as described above.

3.7 Mass spectrometry

For mass spectrometric peptide mapping, 0.5 µl of the sample solution were spotted on a stainless steel target (Bruker Daltonik, Bremen, Germany) and 0.5 µl of DHB matrix solution (12.5 mg/ml in 50 % ACN/0.1 % TFA) were added

and mixed with the sample solution directly on the target. The mixture was then allowed air-dry. MALDI ToF MS measurements were performed using a Reflex III mass spectrometer [Bruker Daltonik, Bremen, Germany] equipped with the SCOUT source and operated in positive ion reflector mode. MALDI was carried out utilizing a nitrogen pulsed laser (337 nm, 3-5 ns pulse width). A total of 400 laser shots were accumulated producing sum spectra ranging from *m/z* 800–3500. Spectra were externally calibrated with a commercially available Peptide Calibration Standard [Bruker Daltonik, Bremen, Germany] then analyzed using FlexAnalysis 2.4 program [Bruker Daltonik, Bremen, Germany]. MS/MS spectra were acquired with an Axima MALDI QIT ToF mass spectrometer [Shimadzu Biotech, Manchester, UK] in positive ion mode utilizing a nitrogen pulsed laser (337 nm, 3-5 ns pulse width) and employing a three-dimensional quadrupole ion trap supplied by helium (pulsed flow gas) for collisional cooling and argon (collisional gas) to impose collisionally induced dissociation (CID) (Koy et al., 2003; Koy et al., 2004). Spectra were externally calibrated with a manually mixed peptide standard consisting of bradykinin (1-7) $[M+H]^+$ 757.39, angiotensin II $[M+H]^+$ 1046.53, angiotensin I $[M+H]^+$ 1296.68, bombesin $[M+H]^+$ 1619.81, *N*-acetyl renin substrate $[M+H]^+$1800.93, ACTH (1-17) $[M+H]^+$ 2093.08, ACTH (18-39) $[M+H]^+$ 2465.19, somatostatin $[M+H]^+$ 3147.46, insulin (oxidized beta chain) $[M+H]^+$ 3494.64. Further processing and analysis of the MS/MS spectra was performed with the Launchpad^{TM} software, version 2.7.1 [Shimadzu Biotech, Manchester, UK].

4. Results

In order to develop and to apply our mass spectrometry-based epitope mapping procedure without immobilization of the antibody we investigated two recombinant proteins, Fibrillarin and RA33, for which monoclonal antibodies with different binding specificities were commercially available. As a prerequisite for the study we first checked antibody binding to the respective full-length antigen by Western blot. The recombinant proteins Fibrillarin and RA33 showed strong bands at apparent masses of 29 kDa and 33 kDa, respectively, showing the decoration of the full-length proteins with the respective antibodies [Figure 1].

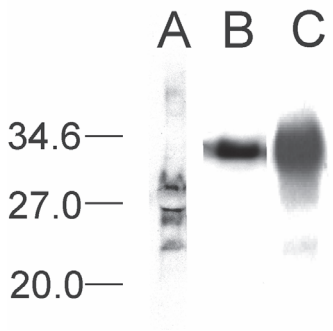


Figure 1: Western Blot analysis. Lane A: Immuno-positive band at 29 kDa apparent molecular mass shows the full length recombinant Fibrillarin protein decorated with anti-His antibody. Bands at 26 kDa and 24 kDa, respectively, show truncations. Lane B: Immuno-positive band at 33 kDa apparent molecular mass shows the full length recombinant RA33 protein decorated with anti-His antibody. Lane C: Immuno-positive band at 33 kDa apparent molecular mass shows the full length recombinant RA33 protein decorated with monoclonal anti-RA33 antibody. Molecular mass marker positions are indicated on the left (numbers refer to apparent molecular masses in kDa).

Fibrillarin, when stained by the monoclonal anti-His-tag antibody, showed also faint bands for truncated forms whereas RA33 staining was producing single bands. All band locations in the Western blot were in agreement with SDS-PAGE analyses of the full-length proteins [*cf*. Figure 2].

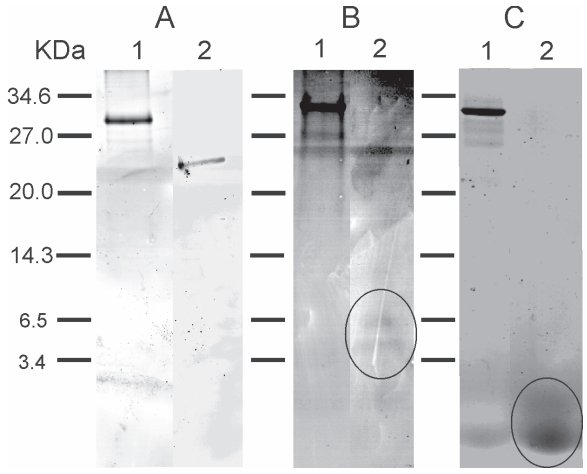


Figure 2: SDS-PAGE of recombinant proteins before and after enzymatic proteolysis. [**A**] 1D SDS-PAGE of recombinant Fibrillarin protein. Lane 1: full length protein. Lane 2: band of trypsin-truncated Fibrillarin. [**B**] 1D SDS-PAGE of recombinant RA33 protein. Lane 1: full length protein. Lane 2: band of trypsin proteolysis products of RA33 are encircled. [**C**]: 1D SDS-PAGE of recombinant RA33 protein. Lane 1: full length protein. Lane 2: bands of thermolysin proteolysis products of RA33 are encircled. Molecular mass marker positions are indicated on the left (numbers refer to apparent molecular masses in kDa).

Next, in-solution proteolytic digests were performed in order to generate peptide mixtures, ideally with (all) peptides possessing molecular masses below 5,000 Da. These mixtures should contain the epitope sequences together with other non-epitope-containing peptides from the same protein or from other (contaminating) proteins in the sample, *e.g*. derived from impurities that may come from the expression systems. The success of the proteolysis reactions was monitored by both, SDS-PAGE [Figure 2] and mass spectrometric peptide mapping [Supplemental figures 1, 2, and 3].

In-solution trypsin digest of Fibrillarin resulted in partial proteolysis where the resulting peptide mixture showed incomplete digestion with a remaining protein band at approx. 24 kDa in the gel [Figure 2A] and with a singly charged molecular ion at *m/z* 23,603 in linear MALDI ToF MS [data not shown], indicating that Fibrillarin is quite resistant to proteolysis.

Nevertheless, in mass spectra ranging from *m/z* 775 to *m/z* 2520 [Table 1; Supplemental Figure 1] plenty of peptide ion signals were observed, resulting in nearly complete sequence coverage of recombinant Fibrillarin [*cf*. Figure 3D].

Table 1: MALDI MS analysis of Fibrillarin peptides after tryptic digest.

Sequence range	$[M+H]^+$ calcd	*m/z* (exp.)
3-27	2676.20	2676.16
35 - 42	981.49	981.49
35 - 50	1979.97	1979.98
43 - 50	1017.49	1017.49
53 - 59	775.42	775.42
60 - 71	1291.65	1291.63
73 - 85	1510.74	1510.74
86 - 91	790.40	790.43
94 - 112	1872.11	1872.13
113 - 145	3416.75	3416.67
149 - 155	786.47	786.47
158 - 168	1240.69	1240.67
175 - 194	2233.14	2233.10
195 - 205	1254.73	1254.73
206 - 215	1071.59	1071.57
216 - 234	1969.92	1969.91
216 - 235	2098.01	2098.01
235 - 254	2520.22	2520.17
236 - 254	2392.13	2392.10
255 - 268	1533.85	1533.87
255 - 270	1761.02	1761.91

As mass spectrometric peptide mapping showed that peptides were produced that covered nearly the entire sequence of Fibrillarin, we considered this mixture suitable for further investigations using size exclusion chromatography despite the fact that digestion was incomplete.

By contrast, tryptic digestion of RA33 resulted in complete proteolysis where the resulting peptide mixture showed ion signals in the mass range between *m/z* 800 and *m/z* 3000 [Table 2; Supplemental Figure 2] leading to nearly full sequence coverage [*cf*. Figure 4D]. Accordingly, SDS PAGE showed faint and diffuse bands with maximal apparent masses of approx. 4 kDa [Figure 2B].

Table 2: MALDI MS analysis of RA33 peptides after tryptic digest.

Sequence range	$[M+H]^+$ calcd (Da)	*m/z* (exp.)
15-20	836.43	836.70
15-21	964.52	964.78
21-37	1927.02	1927.45
22-37	1798.92	1799.33
38-45	1087.48	1087.72
46-53	993.49	993.73
46-58	1491.73	1492.07
62-88	2827.33	2827.95
99-111	1338.70	1338.96
112-119	861.56	861.81
112-128	2008.06	2008.50
113-128	1879.97	1880.39
120-128	1165.52	1165.78
129-136	1050.44	1050.68
129-146	2220.07	2220.10
137-146	1188.65	1188.90
152-167	1851.87	1852.27
152-172	2433.26	2433.77
153-167	1695.76	1696.13
153-172	2277.16	2277.66
173-184	1410.69	1411.02
173-185	1538.78	1539.13
186-199	1648.81	1648.20
190-199	1221.55	1221.84
200-212	1313.60	1313.87
203-212	1013.44	1013.70
213-227	1377.63	1377.94
228-237	912.38	912.63
277-284	781.36	781.61
285-309	2189.91	2190.37

Comparably, in-solution thermolysin digestion of RA33 resulted again in complete proteolysis of the starting material where the resulting peptide mixture showed ion signals in the mass range between *m/z* 600 and *m/z* 3000 [Table 3; Supplemental Figure 3], also leading to nearly full sequence coverage [Figure 5D].

Table 3: MALDI MS analysis of RA33 peptides after thermolytic digest.

Sequence range	$[M+H]^+$ calcd (Da)	*m/z* (exp.)
1-12	1551.63	1550.54
2-12	1420.59	1419.81
2-19	2367.04	2366.54
12-21	1380.70	1380.92
12-26	1867.98	1867.36
30-36	808.36	808.74
30-46	2146.01	2146.43
30-50	2621.18	2621.70
37-46	1372.67 *	1372.70 *
46-62	1890.96	1890.88
50-62	1458.79	1459.57
51-62	1359.73	1360.73
52-64	1464.75	1465.69
53-65	1480.78	1481.64
57-65	1041.56	1041.68
65-76	1303.59	1303.74
80-88	1008.53	1008.78
85-94	1168.68	1168.50
85-95	1239.72	1240.01
96-109	1437.74	1437.79
96-112	1793	1793.62
98-110	1366.71	1366.70
114-130	2044.97	2045.40
119-127	1137.63	1137.69
119-131	1718.78	1718.40
128-137	1319.63	1318.72
132-142	1309.65	1309.60
143-155	1449.79	1449.73
143-156	1596.86	1596.93
157-167	1287.58	1286.68
159-164	745.28	745.87
159-167	1087.47	1086.70
159-169	1299.62	1299.49
160-165	697.28	696.81
160-168	1053.48	1053.31
169-176	1001.58	1002.25
171-176	789.43	789.09
171-181	1282.63	1283.24
176-186	1208.65	1208.52
184-192	1118.61	1118.70
187-205	2106.01	2106.37
187-207	2310.10	2310.25
187-217	3214.48	3214.71
188-206	2139.99	2140.42
193-208	1712.81	1712.66
196-206	1152.55	1151.69
207-218	1127.49	1127.75
207-226	1840.80	1841.19
209-218	923.40	924.16
209-226	1636.71	1637.08
209-239	2890.26	2889.73
218-225	732.33	732.26
219-239	1985.88	1986.24
227-239	1272.57	1272.72
263-280	2002.87	2003.44
280-312	3100.35	3100.89
280-314	3342.47	3342.67
286-312	2482.06	2482.02
286-314	2724.18	2724.79

* oxidation of tryptophan residue

In agreement with mass spectrometry results, SDS PAGE analysis showed faint and diffuse bands with maximal apparent masses of ca. 3.5 kDa [Figure 2C]. Accordingly, both RA33-derived digest mixtures were found well suitable for subsequent size exclusion experiments.

Size exclusion chromatography with a cut-off of roughly 4 to 5 kDa was applied in order to separate high-mass components, such as undigested starting material, protease, and, most importantly the antibody in the digest mixtures from the low-mass components, *i.e.* enzymatically produced peptides. According to the size exclusion principle, high-mass compounds were expected to elute in early fractions (fractions 2 to 5 in our set-up; fraction 1 being the void) and low-mass compounds (below 4 to 5 kDa) should be present in late eluting fractions (fractions 6 to 10 in our set-up). Each experiment was performed such that for control and for investigating the elution profile of the proteolytic peptide mixture alone a first set of 10 fractions was collected with a volume of 10 µl each. Peptides should only be found in the late eluting fractions. In the second set of elution fractions the peptide mixture was first incubated with the antibody and for elution the same experimental conditions were applied as mentioned above. Hence, a comparable set of 10 fractions was obtained. The major difference between the two sets of data should be a shift in migration of the epitope peptide. This peptide was expected to be pulled through the column when bound to the antibody during filtration, now ending up in an early eluting fraction. All fractions from each set were analyzed by MALDI MS in the mass range of *m/z* 800 to *m/z* 3500.

For the control experiment with Fibrillarin, as expected, no peptide ion signals were observed in mass spectra until fraction 5 (early eluting) [Figure 3B] which stands in contrast to the mass spectra that were obtained from higher numbered (late eluting) fractions [data not shown].

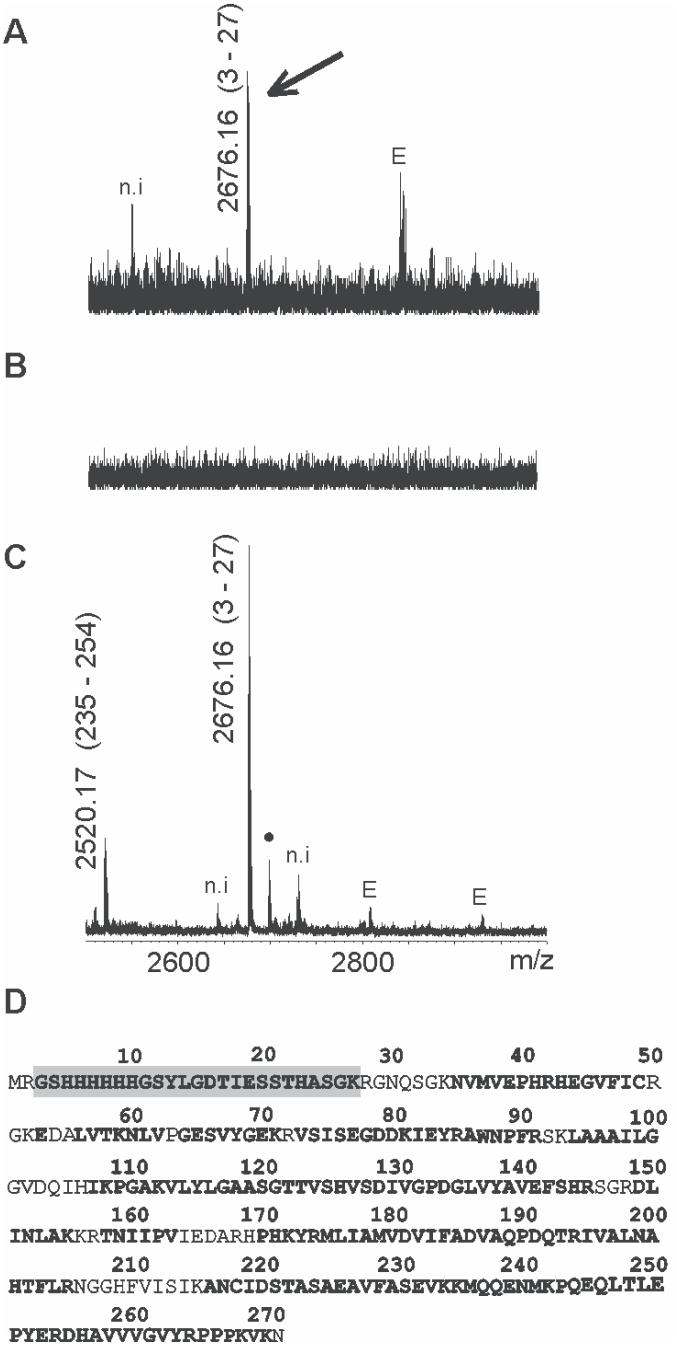


Figure 3: MALDI ToF MS peptide mapping analysis and amino acid sequence of Fibrillarin. [**A**] MS spectrum range *m/z* 2500 – 3000 shows Fibrillarin epitope peptide in early eluting fraction (3) upon binding to anti-His antibody. [**B**] MS spectrum of early eluting fraction in the absence of anti-His antibody remains empty. [**C**] MS spectrum of tryptic Fibrillarin peptides. [**D**] Amino acid sequence in single letter code of Fibrillarin showing sequence coverage after trypsin digest in bold letters. The shaded sequence display the epitope motif that is recognized by the ant-His antibody. Matrix: DHB, n.i: not identified, E: derived from trypsin autoproteolysis, •: sodium adduct.

The elution behaviour of the epitope containing peptide was different in the presence of the monoclonal anti-His antibody during filtration. After incubation with the anti-His antibody the epitope peptide appeared in an early eluting fraction (fraction 3) [Figure 3A]. This epitope peptide at *m/z* 2676.16 which was absent in the control experiment but observed in the peptide mixture before SEC [Figure 3C] was further investigated by MS/MS peptide sequencing and determined the amino acid sequence of the His-tag peptide covering the range aa 3–27 (^{3}GSHHHHHHGSYLGDTIESSTHASGK^{27}) of recombinant Fibrillarin [data not shown] and confirming tryptic cleavage after arginine residue 2 and lysine residue 27.

For RA33 epitope mapping using the monoclonal anti-His-tag antibody, the epitope peptide eluted early (fraction 4) together with its methionine-oxidation products in the presence of the antibody [Figure 4A]. The epitope peptide at *m/z* 2093.13 was found to be covering the sequence range aa 1–16 (^{1}MSHHHHHHHHSMEREK^{16}). Chemically assisted fragmentation-MALDI (CAF-MALDI) and subsequent MS/MS measurements were used to confirm the sequence assignment [data not shown]. This peptide was completely absent in the late eluting fractions after antibody incubation [Figure 4B]. Notably, the peptide ion signal was also not detectable in the peptide mixture before SEC [Figure 4C]. The absence of the ion signal in the latter two samples can be explained by suppression effects and by low ionization yields of the peptide. Only in the rather pure early eluting fraction larger ionization yields were obtained mounting in sufficient intensity of this peptide ion signal to be observable by MS. Again, trypsin was found to cleave the protein after lysine and arginine residues according to its known sequence specificity.

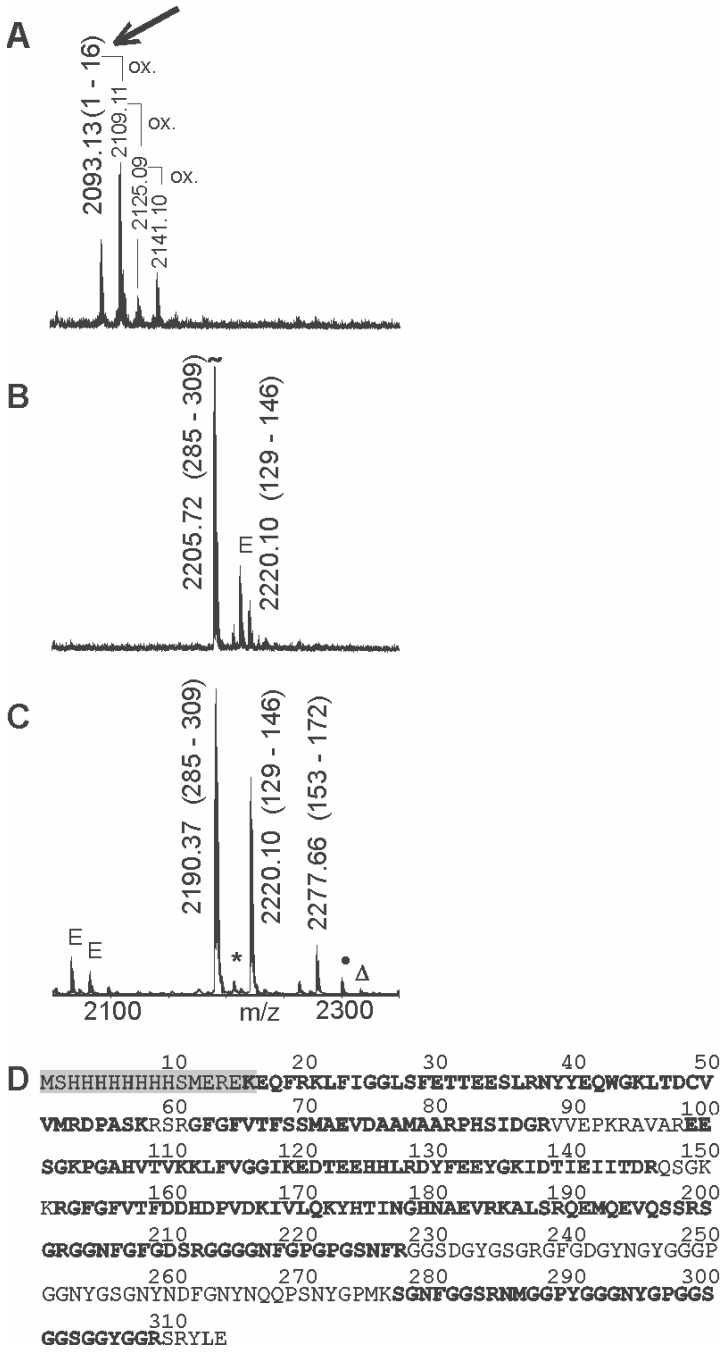


Figure 4: MALDI ToF MS peptide mapping analysis and amino acid sequence of RA33. [**A**] MS spectrum range *m/z* 2050–2350 shows RA33 epitope peptide in early eluting fraction upon incubation with anti-His antibody. [**B**] MS spectrum of RA33 peptides in late eluting fraction upon incubation with anti-His antibody. [**C**] MS spectrum of tryptic RA33 peptides. [**D**] Amino acid sequence in single letter code of RA33 showing sequence coverage after trypsin digest in bold letters. The shaded sequence display the epitope motif that is recognized by the ant-His antibody. Matrix: DHB, E: derived from trypsin autoproteolysis, •: sodium adduct, Δ: potassium adduct, *: oxidation.

Since the developed procedure worked well with two different proteins and reliably isolated the His-tag peptide as the antibody-bound epitope, we went for a first application example and selected a monoclonal antibody that was directed against an internal sequence of the antigen RA33. Again, RA33 was first digested, this time with thermolysin. Thermolysin is a "non-sequence specific" enzyme which preferentially cleaves at the *N*-terminal side of bulky and aromatic residues Ile, Leu, Val, Ala, Met, and Phe (Keil, 1992), but was readily applicable in our experiments as it yielded in complete proteolysis, which is advantageous when the goal is to determine a specific epitope to the smallest number of amino acids residues, as long as the epitope is not destroyed by cleavage. The resulting epitope peptide eluted early (fraction 3) in the presence of the antibody [Figure 5A]. In the control experiment, *i.e.* in the absence of the antibody during filtration, the same peptide eluted late (fraction 8) together with other non-epitope peptides [Figure 5B]. For comparison, the mass spectrum of the thermolytic peptide mixture before SEC is shown [Figure 5C]. This epitope peptide at *m/z* 1240.01 was further investigated using MS/MS peptide sequencing [Figure 6] which confirmed that the peptide comprised the range aa 85–95 (^{85}IDGRVVEPKRA^{95}) of RA33. This result stands in agreement with the previously assigned epitope using immuno-analytical methods coupled with mass spectrometry (El-Kased et al., 2009).

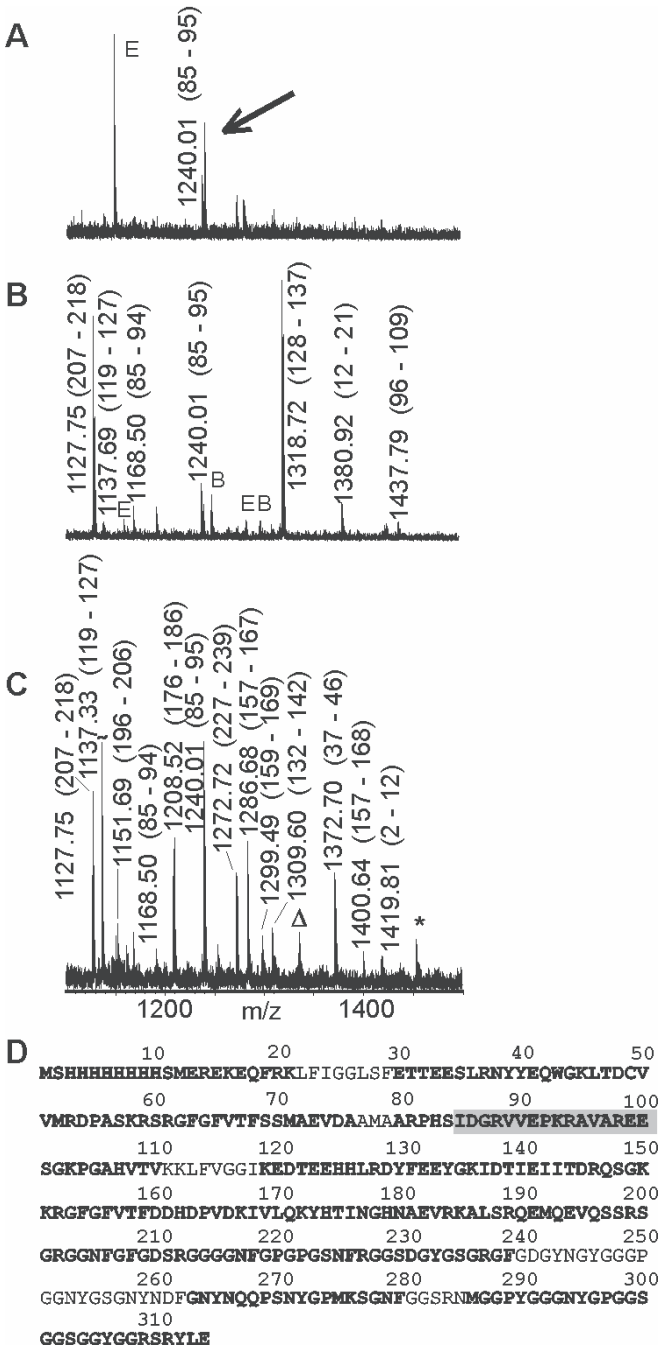


Figure 5: MALDI ToF MS peptide mapping analysis and amino acid sequence of RA33. [**A**] MS spectrum range *m/z* 1100–1500 shows RA33 epitope peptide in early eluting fraction upon incubation with monoclonal anti-RA33 antibody. [**B**] MS spectrum of late eluting RA33 peptides in the absence of monoclonal anti-RA33 antibody. [**C**] MS spectrum of thermolytic RA33 peptides. [**D**] Amino acid sequence in single letter code of RA33 showing sequence coverage after thermolysin digest in bold letters. The shaded sequence displays the epitope motif that is recognized by the monoclonal anti-RA33 antibody. Matrix: DHB, E: derived from thermolysin autoproteolysis, Δ: potassium adduct, B: derived from buffer, *: oxidation.

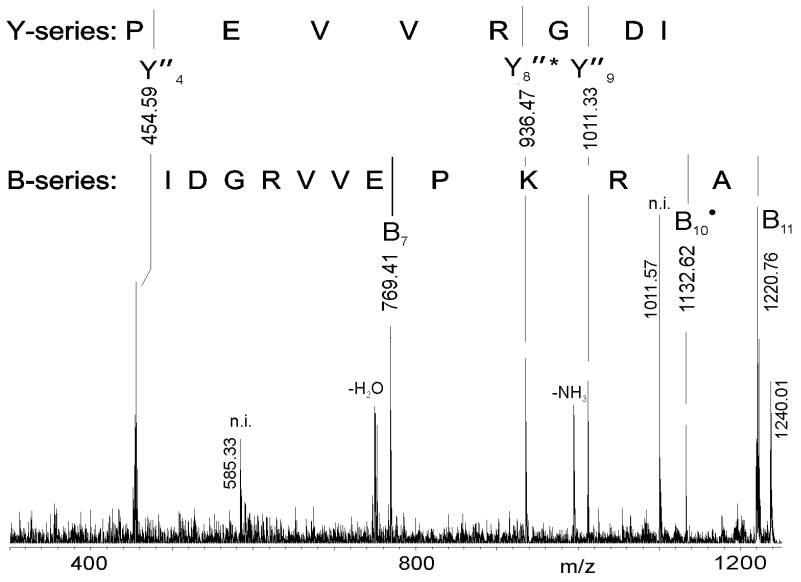


Figure 6: MALDI QIT ToF MS/MS spectrum of the ion signal at *m/z* 1240.01 (*c.f* Figure 5A). The determined partial amino acid sequence is depicted and was assigned to an RA33 peptide comprising amino acids 85–95. The mass spectrometric fragment ions from the Y″ type ion series and the B type ion series are indicated. Loss of ammonia and loss of water, respectively, is indicated. DHB was used as a matrix.

The here described experiments were accompanied by control runs in which all conditions of the size exclusion chromatography steps were tested in order to determine potentially interfering ion signals that may appear in mass spectra after elution (i) due to bleeding of the gel slurry during filtration [Supplemental Table 1], (ii) due to background signals derived from enzyme autoproteolysis during incubation [Supplemental Tables 2 and 3], or (iii) derived from partial degradation of the antibodies used in the study [Supplemental Table 4]. The generated lists of background signals from these mock experiments were found helpful for defining the epitope peptide ion signals in the early eluting fractions after subtraction of all potential background signals.

In the end, the most important criterion for defining the epitope peptide was the shift in elution of a peptide upon binding to the antibody so that it was identified in the early eluting fraction where otherwise only high mass compounds appeared, despite being small enough to elute in late eluting fractions when unbound.

5. Discussion

Fibrillarin, is a 34 kDa autoantigen, present in all nucleated cell types that derives its name from its localization to both the fibrillar center (FC) and dense fibrillar component (DFC) of the nucleolus (Ochs RL 1985). As a component of

all small nucleolar ribonucleoprotein particles (snoRNPs), Fibrillarin seems to be involved in nearly all major post-transcriptional activities in ribosome synthesis, the first steps of rRNA processing, pre-rRNA modification, and ribosome assembly (Tollervey et al., 1993). Fibrillarin is a target for the spontaneously arising auto-antibodies in systemic sclerosis autoimmune disease (Ssc; scleroderma) (Takeuchi et al., 1995). Similarly, the RA33 auto-antigen, present in rheumatic autoimmune diseases, carries its name according to its migration behaviour in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and has been found to be identical to the protein named hnRNP A2/B1 (RefSeq accessions NP_002128 and NP_112533; Uniprot Accession number P22626). HnRNP proteins are associated with pre-mRNAs in the nucleus in complex 4 which forms part of the spliceosome (Steiner and Smolen, 2002). They are involved in pre-mRNA processing and other aspects of mRNA metabolism and transport. It has been reported that the presence of anti-RA33 antibodies may preceed disease symptoms by one year (Steiner et al., 1996). Therefore they can provide diagnostic help early in disease, particularly when Rheumatoid factor (RF) is negative in patient samples. In conjunction with anti-cyclic citrullinated peptide antibody (antiCCP antibody) level determination, measuring anti-RA33 autoantibody levels in RA patients and in other autoimmune patients has become important in particular for diagnosing RA in the absence of RF and for estimating severity of the disease (Feist et al., 2007; Steiner and Smolen, 2002). As both proteins play their roles in autoimmune diseases it is of interest to identify epitopes to which autoantibodies from individual patients are directed. Therefore, epitope mapping approaches that are suitable for studying patient sample-derived autoantibodies are of high interest.

The functional integrity of an antibody in all steps of our epitope mapping procedure is a prerequisite for successful application. This was checked by dot blot analysis with fractions from size exclusion chromatography. Binding of the antibody to the antigen was found in early eluting fractions [Supplemental Figure 6], showing that the antibody survived the procedure intact and biologically active. The widely accepted knowledge that antibodies in general are resistant to proteolytic digestion, however, appeared not to be absolute. We recommend to check each commercially available antibody by incubation with the protease of choice, as in one example the anti-His-tag antibody was partially digested generating two dominant peptide ion signals at *m/z* 1488.73 and *m/z* 2383.41 upon incubation with trypsin [Figure 7].

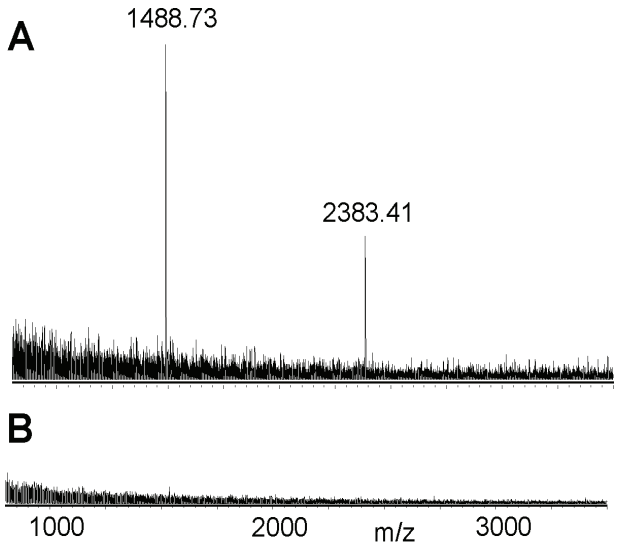


Figure 7: MALDI ToF MS spectrum of antibody derived peptides in early fraction after incubation with protease. [**A**] MS spectrum range *m/z* 750–3500 of the early eluting fraction of anti-His antibody after incubation with trypsin. [**B**] MS spectrum of the early eluting fraction of monoclonal antibody after incubation with thermolysin. DHB was used as a matrix.

Both peptides were analyzed further using mass spectrometric peptide sequencing and confirmed that the generated peptides were part of the IgG heavy chain variable region and of the IgG light chain variable region, respectively [data not shown]. The proteolytically nicked antibody subsequently lost its capability to bind to its antigen and, hence, was not applicable for epitope mapping. By contrast, a different and more robust anti-His-tag antibody as well as the monoclonal anti-RA33 antibody proved to be stable against proteolysis under the employed conditions and, therefore, both antibodies were found suitable for our peptide mapping procedure.

Preferably for our method, the proteolytic enzyme of choice should produce peptides of masses below 4 kDa to ensure a shift in elution upon binding to the antibody as compared to the free peptide. At the same time, the presence of high-mass compounds do not interfere with the epitope mapping procedure as long as they do not contain all of the epitope sequences. This is due to the fact that the mass spectrometric measurements were tuned to the (low) mass range of interest below *m/z* 4,000. Because with peptides in that mass range MS/MS peptide fragmentation normally can be readily performed using the epitope peptides from the early eluting fractions, sequence specificity of the protease is not a major concern in epitope mapping using our method.

Estimating the adequate sample amount is an important step for obtaining

reliable results with this size exclusion chromatography-based method. Sample overloading leads to the escape of peptides to early fractions. In our approach using a sample volume of ca. 4% of the total bed volume and a peptide amount up to 35 pmol was found adequate to obtain optimal elution profiles. Our experimental procedure shows that incubation at a 3:1 ratio of antibody to peptides is well suitable for epitope mapping. This rather fixed antibody/peptide ratio differs from those reported in the literature where excess of antibody varies in broader ranges (Pimenova et al., 2009*;* Dhungana et al., 2009; Zhao and Chait, 1994) while in other studies peptides were used in excess (Bílková et al., 2005; Parker and Tomer, 2002; Peter and Tomer, 2001; Kiselar and Downard, 1999; Papac et al., 1994).

With our method dissociation of the antigen-antibody complex prior to mass spectrometric analysis is unnecessary because dissociation occurs during sample preparation of the collected fraction material onto the MALDI target simultaneously to the crystallization process of the MALDI matrix, leading to a decrease in sample handling and making it suitable for screening purposes in a clinical environment. We are ultimately aiming at a screening procedure to identify major epitopes in patients that may be suitable in the future for stratification of patients. Hence, reproducibility, ease of handling, minimal sample consumption as well as robustness of the method, as is the case with our method, are of utmost importance.

6. Acknowledgements

We thank M. Sieb, T. Mittag and J. Taubenheim for excellent technical assistance. This work has been supported by a grant of the State of Western Pomerania "Future Fonds: Research of Excellence; FKZ: UR 08 051".

7. References

Bílková, Z., Stefanescu, R., Cecal, R., Korecká, R., Ouzká, Š., Ježová, J., Viovya, J.-L., and Przybylski, M. (2005). Epitope extraction technique using a proteolytic magnetic reactor combined with Fourier-transform ion cyclotron resonance mass spectrometry as a tool for the screening of potential vaccine lead peptides. Eur J Mass Spectrom 11, 489-495.

Brady, L.J., Valliere-Douglass, J., Martinez, T., and Balland, A. (2008). Molecular Mass Analysis of Antibodies by On-Line SEC-MS. J Am Soc Mass Spectrom 19, 502-509.

Burnens, A., Demotz, S., Corradin, G., Binz, H., and Bosshard, H. (1987). Epitope mapping by chemical modification of free and antibody-bound protein antigen. Science 235, 780-783.

Dhungana, S., Williams, J.G., Fessler, M.B., and Tomer, K.B. (2009). Epitope Mapping by Proteolysis of Antigen–Antibody Complexes. In Epitope Mapping Protocols, M. Schutkowski, and U. Reineke, eds. (Humana Press), pp. 87-101.

El-Kased, R.F., Koy, C., Deierling, T., Lorenz, P., Qian, Z., Li, Y., Thiesen, H.-J., and Glocker , M.O. (2009). Mass Spectrometric and Peptide Chip Epitope Mapping of Rheumatoid Arthritis Autoantigen RA33 Eur J Mass Spectrom 15, 747-759.

Feist, E., Egerer, K., and Burmester, G.R. (2007). Autoantikörperprofile bei der rheumatoiden Arthritis. Z Rheumatol 66, 212-218.

Gonzalez, G., ÖRn, A., Cazzulo, J.J., and GrÖNvik, K.O. (1994). Production and Characterization of Monoclonal Antibodies Against the Major Cysteine Proteinase of Trypanosoma cruzi. Scand J Immunol 40, 389-394.

Hager-Braun, C., Katinger, H., and Tomer, K.B. (2006). The HIV-neutralizing monoclonal antibody 4E10 recognizes N-terminal sequences on the native antigen. J Immunol 176, 7471-7481.

Hager-Braun, C., and Tomer, K.B. (2005). Determination of protein-derived epitopes by mass spectrometry. Expert Rev Proteomics 2, 745-756.

Hochleitner, E.O., Borchers, C., Parker, C., Bienstock, R.J., and Tomer, K.B. (2000). Characterization of a discontinuous epitope of the human immunodeficiency virus (HIV) core protein p24 by epitope excision and differential chemical modification followed by mass spectrometric peptide mapping analysis. Protein Sci 9, 487-496.

Jemmerson, R. (1996). Epitope Mapping by Proteolysis of Antigen-Antibody Complexes. In Epitope Mapping Protocols, G. E. Morris, ed. (Humana Press), pp. 97-108.

Juszczyk, P., Paraschiv, G., Szymanska, A., Kolodziejczyk, A.S., Rodziewicz-Motowidlo, S., Grzonka, Z., and Przybylski, M. (2009). Binding epitopes and interaction structure of the neuroprotective protease inhibitor cystatin C with beta-amyloid revealed by proteolytic excision mass spectrometry and molecular docking simulation. J Med Chem 52, 2420-2428.

Kazemi, M., and Finkelstein, R.A. (1991). Mapping epitopic regions of cholera toxin B-subunit protein. Mol Immunol 28, 865-876.

Keil, B. (1992). Specificity of proteolysis. Berlin - Heidelberg - New York: Springer-Verlag, 335.

Kiselar, J.G., and Downard, K.M. (1999). Direct Identification of Protein Epitopes by Mass Spectrometry without Immobilization of Antibody and Isolation of Antibody-Peptide Complexes.

Anal Chem 71, 1792-1801.

Koy, C., Mikkat, S., Raptakis, E., Sutton, C., Resch, M., Tanaka, K., and Glocker, M.O. (2003). Matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight mass spectrometry sequencing resolves structures of unidentified peptides obtained by in-gel tryptic digestion of haptoglobin derivatives from human plasma proteomes. Proteomics 3, 851-858.

Koy, C., Resch, M., Tanaka, K., and Glocker, M.O. (2004). Primary structure details of haptoglobin a chain proteins from human plasma samples are resolved by QIT ToF MSn sequencing. Eur J Mass Spectrom 10, 393-399.

Lazar, A.C., Wang, L., Blättler, W.A., Amphlett, G., Lambert, J.M., and Zhang, W. (2005). Analysis of the composition of immunoconjugates using size-exclusion chromatography coupled to mass spectrometry. Rapid Commun Mass Spectrom 19, 1806-1814.

Logan, A.J., Williamson, E.D., Titball, R.W., Percival, D.A., Shuttleworth, A.D., Conlan, J.W., and Kelly, D.C. (1991). Epitope mapping of the alpha-toxin of Clostridium perfringens. Infect Immun 59, 4338-4342.

Macht, M., Fiedler, W., Kürzinger, K., and Przybylski, M. (1996). Mass spectrometric mapping of protein epitope structures of myocardial infarct markers myoglobin and troponin T. Biochemistry 35, 15633-15639.

Manca, F. (1991). Interference of monoclonal antibodies with proteolysis of antigens in cellular and in acellular systems. Ann Ist Super Sanita 27, 15-19.

Nelson, P.N., Reynolds, G.M., Waldron, E.E., Ward, E., Giannopoulos, K., and Murray, P.G. (2000). Demystified monoclonal antibodies. Mol Pathol 53, 111-117.

Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9, 255-262.

Ochs RL , L.M., Spohn WH , Busch H (1985). Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell 54, 123-133.

Papac, D.I., Hoyes, J., and Tomer, K.B. (1994). Epitope mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex by proteolysis followed by matrix-assisted laser desorption ionization mass spectrometry. Protein Sci 3, 1485-1492.

Parker, C., Papac, D., Trojak, S., and Tomer, K. (1996). Epitope mapping by mass spectrometry: determination of an epitope on HIV-1 IIIB p26 recognized by a monoclonal antibody. J Immunol 157, 198-206.

Parker, C., and Tomer, K. (2002). MALDI/MS-based epitope mapping of antigens bound to immobilized antibodies. Mol Biotechnol 20, 49-62.

Parker, C.E., and Tomer, K.B. (2000). Epitope Mapping by a Combination of Epitope Excision and MALDI-MS. In Mass Spectrometry of Proteins and Peptides, J. R. Chapman, ed. (Humana Press), pp. 185-201.

Peter, J.F., and Tomer, K.B. (2001). A General Strategy for Epitope Mapping by Direct MALDI-TOF Mass Spectrometry Using Secondary Antibodies and Cross-Linking. Anal Chem 73, 4012-4019.

Pimenova, T., Meier, L., Roschitzki, B., Paraschiv, G., Przybylski, M., and Zenobi, R. (2009).

Polystyrene beads as an alternative support material for epitope identification of a prion-antibody interaction using proteolytic excision–mass spectrometry. Anal Bioanal Chem 395, 1395-1401.

Przybylski, M. (1994). Mass spectrometric approaches to the characterization of tertiary and supramolecular structures of biomolecules. Adv Mass Spectrom 13, 257–283.

Saint-Remy, J. (1997). Epitope mapping: a new method for biological evaluation and immunotoxicology. Toxicology 119, 77-81.

Schägger, H., Aquila, H., and Von Jagow, G. (1988). Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis. Anal Biochem 173, 201-205.

Steiner, G., Skriner, K., Hassfeld, W., and Smolen, J.S. (1996). Clinical and immunological aspects of autoantibodies to RA33/hnRNP-A/B proteins-a link between RA, SLE and MCTD. Mol Biol Rep 23, 167-171.

Steiner, G., and Smolen, J.S. (2002). Neue Autoantikörper in der Diagnostik der rheumatoiden Arthritis. Z Rheumatol 61, 667-673.

Suckau, D., Kohl, J., Karwath, G., Schneider, K., Casaretto, M., Bitter-Suermann, D., and Przybylski, M. (1990). Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping. Proc Natl Acad Sci U S A 87, 9848-9852.

Takeuchi, K., Turley, S., Tan, E., and Pollard, K. (1995). Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity. J Immunol 154, 961-971.

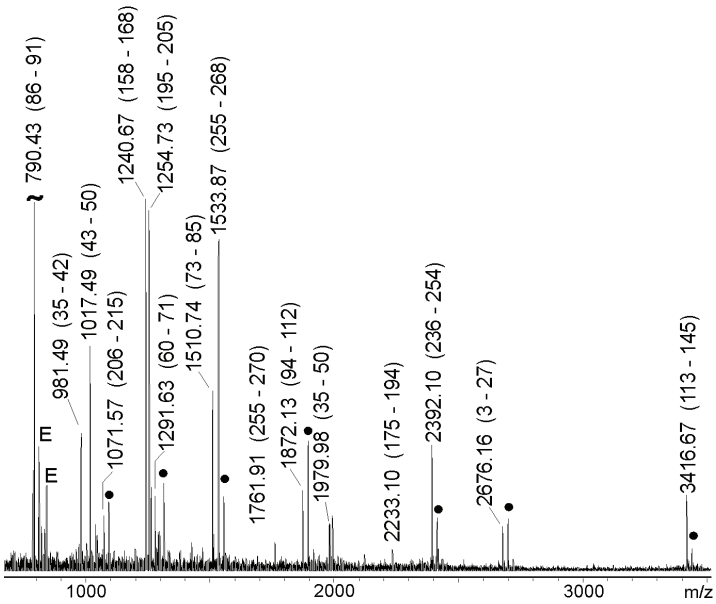
Tian, X., Maftei, M., Kohlmann, M., Allinquant, B., and Przybylski, M. (2007). Differential epitope identification of antibodies against intracellular domains of alzheimer's amyloid precursor protein using high resolution affinity-mass spectrometry. In Subcellular Biochemistry, E. Bertrand, and M. Faupel, eds. (The Netherlands, Springer), pp. 339-354.

Tollervey, D., Lehtonen, H., Jansen, R., Kern, H., and Hurt, E.C. (1993). Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. Cell 72, 443-457.

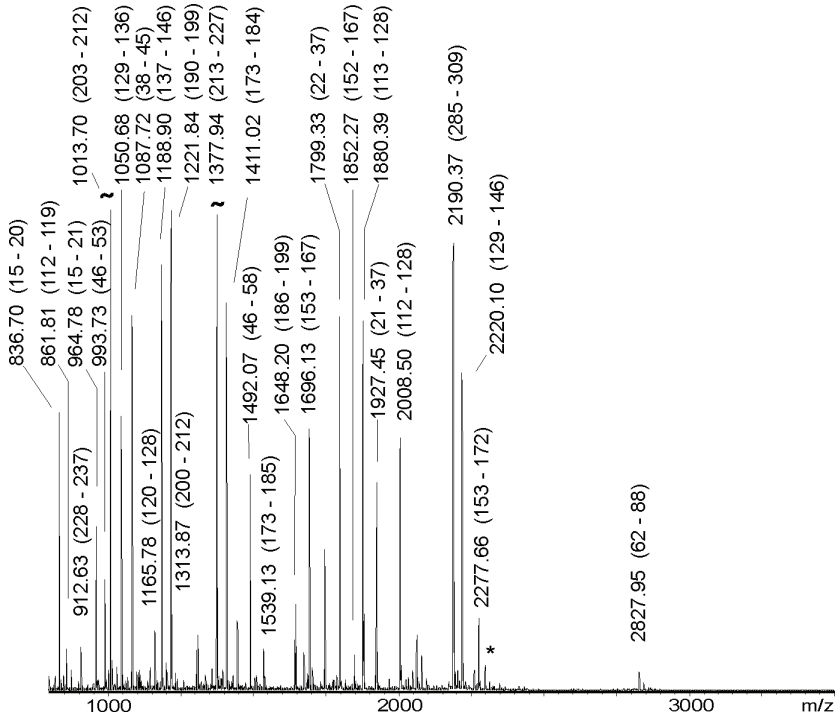
Zhao, Y., and Chait, B.T. (1994). Protein Epitope Mapping By Mass Spectrometry. Anal Chem 66, 3723-3726.

Zhao, Y., Muir, T.W., Kent, S.B., Tischer, E., Scardina, J.M., and Chait, B.T. (1996). Mapping protein-protein interactions by affinity-directed mass spectrometry. Proc Natl Acad Sci U S A 93, 4020-4024.

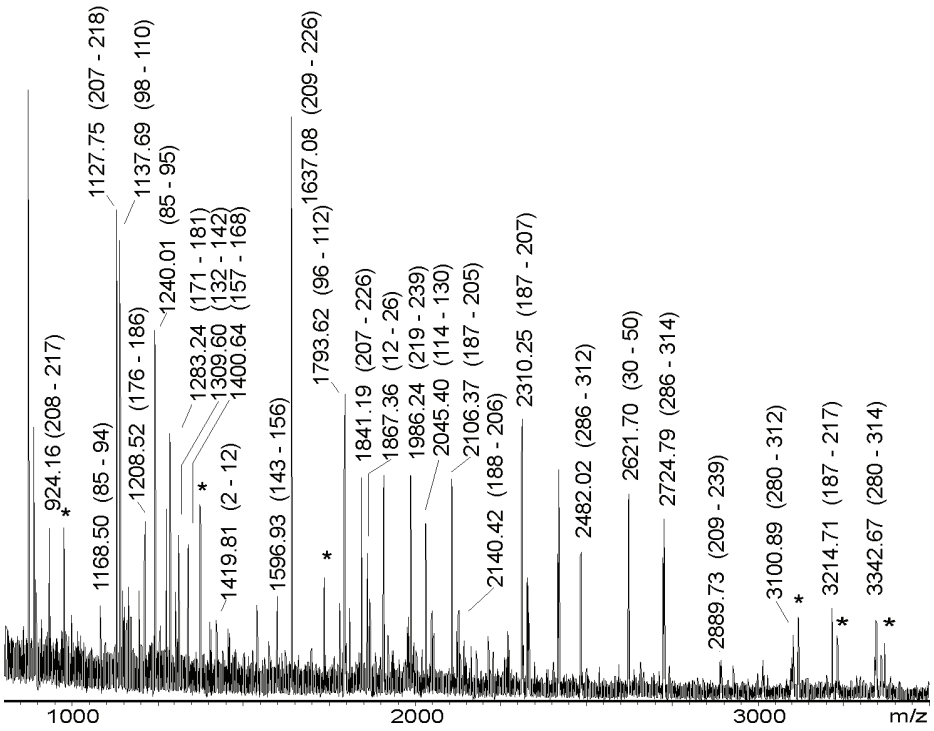
8. Supplemental material



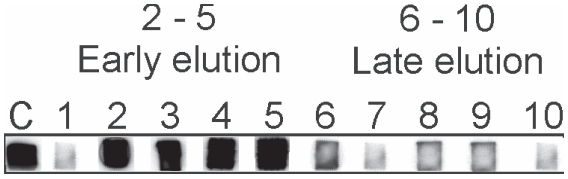
Supplemental Figure 1: MALDI ToF MS analysis of tryptic digest of Fibrillarin. Peptide ion signals are labeled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses. E: ion signals due to trypsin enzyme autoproteolysis. A dot symbol indicates sodium adducts. Matrix: DHB.



Supplemental Figure 2: MALDI ToF MS analysis of tryptic digest of RA33. Peptide ion signals are labeled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses. The asterisk indicates oxidation. Matrix: DHB.



Supplemental Figure 3: MALDI ToF MS analysis of thermolytic digest of RA33. Peptide ion signals are labeled with *m/z* values. Matching amino acid sequence ranges are shown in parentheses. The asterisk indicates oxidation. Matrix: DHB.



Supplemental Figure 4: Dot blot analysis of monoclonal antibody against RA33 after incubation with thermolysin and after size exclusion chromatography. Numbers indicate eluting fractions. . C: control reaction with monoclonal antibody without elution. Fractions 1 and 6–10 show only background staining.

Supplemental Table 1: Exclusion list of ion signals observed after size exclusion chromatography of buffer solution.

m/z (exp.)
868.78
905.48
997.43
1052.32
1126.36
1148.83
1246.93
1295.81
1535.76
1590.23
1672.14
1701.28
1718.31
1803.36
1877.30
1950.44
1992.49
2107.56
2347.50
2764.47

Supplemental Table 2: Exclusion list of ion signals observed after size exclusion chromatography of trypsin autoproteolysis products.

m/z (exp.)
842.54
856.48
984.41
1020.43
1044.46
1189.42
1432.52
1446.57
1607.60
1621.58
1940.60
2210.86
2224.84
2238.83
2845.24
2928.26

Supplemental Table 3: Exclusion list of ion signals observed after size exclusion chromatography of thermolysin autoproteolysis products.

m/z (exp.)
973.37
1007.40
1032.46
1136.45
1152.45
1158.46
1174.32
1208.55
1283.55
1335.65
1400.67
1448.75
1495.68
1824.95
1866.90
1986.58
2029.96
2070.46
2365.33

Supplemental Table 4: Exclusion list of ion signals observed after size exclusion chromatography of anti-His antibody incubated with trypsin.

m/z (exp.)
935.18
1017.33
1083.30
1124.35
1254.38
1232.44
1274.48
1288.41
1388.47
1398.48
1567.49
1593.59
1737.67
1809.58
1865.80
1872.76
1926.70
2027.85
2054.77
2187.86

m/z (exp.)
2440.86
2587.97
2610.05
2652.05
2846.09
3078.25
3339.49

Curriculum Vitae

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WS 2007/2008 & SS 2008

Guest student at Medical Faculty where I performed my masters thesis at Proteome Center Rostock, University of Rostock.

February 2001 - January 2007

Working at Dar El-Shefa Hospital, Ministry of health, Cairo, Egypt.

June 2000 - October 2005

Working as a Pharmacist at a private pharmacy, Cairo, Egypt.

Training

Training at a Multinational Company (PFIZER Egypt) at the production sector and as a Medical Representative.

Training at the Arab Pharmaceutical Company at both the Production and Quality Control sectors.

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Education

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Medical faculty seminar course 31311

“Aktuelle Forschungsprojekte der Medizininischen Fakultat” at Medical faculty, Rostock University.

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SS2009

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Immunology seminar course 31305

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Medical faculty seminar course 30013

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WS 2007/2008 and SS 2008

Master thesis at Proteome Center, Rostock University. under supervision of Professor Dr. Michael O.Glocker, Co-supervisor: Professor Dr.Alaa El-Dein Shawky.

Proteomics seminar course 31324

"Proteomics- Neue Ansatze zur Aufklarung genregulatorischer Netzwerke" at Medical faculty, Rostock University.

Immunology seminar course 31320

"Aktuelle Aspekte der Immunologie" at Medical faculty, Rostock University.

2004 – 2006

Master degree courses in Microbiology and Immunology with final grade "Very Good", Faculty of Pharmacy, Cairo University.

Subjects included:

Molecular biology and advanced genetics of microorganisms.

Advanced immunology and immunological products.

Microbial resistance to antibiotics and evaluation of antimicrobials and antibiotics. Sterilisation validation and Quality control of sterile products.

Basic statistics.

Computer and its applications.

Literature search and Scientific English language course.

1995 – 2000

Bachelor degree in Pharmaceutical Sciences, Faculty of Pharmacy, Cairo University.

1992 – 1995

Secondary school certificate, Science division, Saint Fatima English Language Schools, Cairo, Egypt.

Practical experience

Epitope mapping using mass spectrometric analysis (epitope extraction and epitope excision).

Mass spectrometric analysis of peptides and proteins (MALDI- ToF PMF, MALDI- ToF MS, ESI MS, and ISD MALDI- ToF MS).

Performing Gel electrophoresis.

High performance liquid chromatography (HPLC) and affinity chromatography.

Western blot analysis and dot blot technique.

Size exclusion chromatography.

Computer and software experience

Working with data analysis programs; CorelDraw, GPMAW, Flex analysis, Masslynx, Biotools, Progenesis softwares and different protein databases.

Good computer skills; Word, Excel, PowerPoint and Internet.

List of Publications

Mass Spectrometric and Peptide Chip Epitope mapping of Rheumatoid Arthritis Autoantigen RA33.

R. F. Elkased, C. Koy, T. Deierling, P. Lorenz, Z. Qian, Y. Li, H.-J. Thiesen and M. O. Glocker, Eur. J. Mass Spectrom., ***15***, 747-759 (2009).

Mass Spectrometric and Peptide Chip Epitope Analysis on the RA33 Autoantigen with Sera from Rheumatoid Arthritis Patients.

R. F. Elkased, C. Koy, P. Lorenz, S. Drynda, R. Guthke, Z. Qian, D. Koczan, Y. Li, J. Kekow, H.-J. Thiesen and M. O. Glocker, Eur. J. Mass Spectrom., ***16***, 443-451 (2010).

A Novel Mass Spectrometric Epitope Mapping Approach Without Immobilization of the Antibody

R. F. El-Kased , C. Koy , P. Lorenz , H. Montgomery , K. Tanaka, H.-J. Thiesen, and M. O. Glocker. J. Proteomics Bioinform, ***4***, 001-009 (2011).

Mass Spectrometric Epitope Mapping of Rheumatoid Arthritis Autoantigen RA33.

R. F. Elkased, C. Koy, T. Deierling, P. Lorenz, Z. Qian, Y. Li, H.-J. Thiesen, and M. O. Glocker. 18th International Mass Spectrometry Conference 2009, Bremen, Germany. (Poster Presentation)

Mass Spectrometric and Peptide Chip Epitope Analysis of Autoantigens and Application to Diagnostics.

R. F. Elkased, C. Koy, T. Deierling, P. Lorenz, S. Drynda, Z. Qian, Y. Li, J. Kekow, H.-J. Thiesen, and M. O. Glocker. HUPO VIII World Congress 2009, Toronto, Canada. (Poster Presentation)

In-solution Epitope Mapping.

Reham El-Kased, Cornelia Koy, Peter Lorenz, Johannes Wollbold, Felix Steinbeck, Hans-Jürgen Thiesen, and Michael O. Glocker. 10th German Peptide Symposium 2011, Berlin, Germany.