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Epitope mapping of the U1 small nuclear ribonucleoprotein particle in patients with systemic lupus erythematosus and mixed connective tissue disease

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Abstract

Systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) are autoimmune illnesses characterized by the presence of high titers of autoantibodies directed against a wide range of ‘self’ antigens. Proteins of the U1 small nuclear ribonucleoprotein particle (U1 snRNP) are among the most immunogenic molecules in patients with SLE and MCTD. The recent release of a crystallized U1 snRNP provides a unique opportunity to evaluate the effects of tertiary and quaternary structures on autoantigenicity within the U1 snRNP. In the present study, an epitope map was created using the U1 snRNP crystal structure. A total of 15 peptides were tested in a cohort of 68 patients with SLE, 29 with MCTD and 26 healthy individuals and mapped onto the U1 snRNP structure. Antigenic sites were detected in a variety of structures and appear to include RNA binding domains, but mostly exclude regions necessary for protein–protein interactions. These data suggest that while some autoantibodies may target U1 snRNP proteins as monomers or apoptosis-induced, protease-digested fragments, others may recognize epitopes on assembled protein subcomplexes of the U1 snRNP. Although nearly all of the peptides are strong predictors of autoimmune illness, none were successful at distinguishing between SLE and MCTD. The antigenicity of some peptides significantly correlated with several clinical symptoms. This investigation implicitly highlights the complexities of autoimmune epitopes, and autoimmune illnesses in general, and demonstrates the variability of antigens in patient populations, all of which contribute to difficult clinical diagnoses.

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Introduction

A prominent feature of systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD), is the presence of high titers of serum antibodies targeting diverse nuclear autoantigens, including components of the U1 small nuclear ribonucleoprotein particle (snRNP).^{1–3} The U1 snRNP comprises the U1 small nuclear RNA, onto which 10 proteins bind (U1A, U1C, U1-70K, SmB/B', SmD1, SmD2, SmD3, SmE, SmF and SmG).⁴

Typically, autoantibodies directed against U1A, U1C and U1-70K proteins of the U1 snRNP and the U1 snRNA itself are mainly associated with MCTD, whereas autoantibodies directed against the Sm complex (SmD1, SmD2, SmD3, SmE, SmF and SmG) are more frequently seen in patients suffering from SLE;⁵ however, there is often a significant overlap between the autoantibodies produced in patients with SLE and MCTD.^{6,7} Given the substantial similarities of clinical symptoms, proper diagnoses of SLE and MCTD can often be challenging.

There is considerable evidence that increased apoptosis, coupled with impaired clearing of dead cell constituents, may contribute to the onset of the rheumatic autoimmune disorders.^{8–10} The release of nuclear components following apoptosis-induced cell lysis can cause antibodies to come into contact with molecules that would otherwise be protected by the cytoplasmic membrane and the nuclear envelope. In addition, apoptosis-associated, proteolytic digestion and other modifications to 'self' molecules can generate novel epitopes to which the antibody repertoire can identify and respond. For example, the SmF protein has been shown to undergo apoptosis-linked, proteolytic digestion,¹¹ and cleavage of U1-70K by apoptotic proteases causes a stronger autoimmune reaction than native U1-70K in patients with MCTD.^{12,13} The present study was designed to examine the complexities of autoimmune responses against the U1 snRNP in SLE and MCTD patients. To this end, 15 synthetic octa-peptides from all the proteins of the U1 snRNP were tested in a cohort of sera from patients with SLE or MCTD, in addition to healthy individuals. The study reveals striking variability in antigenicity among patients within a single disease and even in the same individual at different times. Furthermore, given the considerable overlap in reactivity between SLE and MCTD patients, none of the peptides were able to significantly distinguish between the two illnesses. Interestingly, an epitope map using the crystal structure of the U1 snRNP illustrates the extensive diversity in both the position and conformation of U1 snRNP epitopes. Moreover, this study suggests that characteristics other than antibody accessibility may play a role in antigenicity, which supports the hypothesis that autoantibodies directed against the U1 snRNP are recognizing potentially novel epitopes subsequent to protease degradation, phosphorylation or other modifications.

Materials and methods

Characterization of patient samples

A total of 156 serum samples from patients diagnosed with SLE (82 samples from 68 individuals), MCTD (42 samples from 29 patients) and healthy individuals (32 samples from 26 persons) were obtained from whole blood collected at time of recruitment and follow-up visits (when applicable) as previously described.¹⁴ Patient sample characterization included complete blood count, routine blood chemistry and serological testing for the presence of anti-nuclear antibodies (ANA), anti-cardiolipin antibodies (ACA), and reactivity against double-stranded-DNA (dsDNA), U1 ribonucleo-protein complex (RNP), Smith antigen (Sm), SSA/Ro, SSB/La and Scl-70 (Table 1 and Supplementary file 1) as previously described.¹⁴ Commercial laboratory tests were run by Quest Diagnostics Incorporated. All methods were performed according to the Florida International University and the University of Miami Institutional Review Board (IRB) accepted protocols (IRB numbers: 040308-00 for the former and 20030724 and 20040286 for the latter).

Preparation of the U1-70K antigen and anti-U1-70K enzyme-linked immunosorbent assays

A peptide spanning amino acids 63-205 of U1-70K and encompassing the RNA-binding domain of the protein was expressed in *Escherichia coli* from the pMAL-c2G plasmid (New England Biolabs, Ipswich, MA, USA) as a maltose-binding protein (MBP) fusion protein and affinity purified over amylose columns as previously described.¹³ The identity and purity of the product was confirmed by immunoblotting using standard anti-U1-70K sera (data not shown). Enzyme-linked immunosorbent assays (ELISAs) were performed in duplicates, and standard positive and negative control sera were included on each plate.¹³ Briefly, 96-well, flat-bottom microtiter plates (Corning Incorporated, Lowell, MA, USA) were incubated overnight at 4°C with purified antigen in phosphate buffered saline (PBS). After washing with PBS-0.05% Tween-20 (PBS-T) buffer, plates were blocked with 1% bovine serum albumin (BSA) in PBS-T, incubated with human sera at final dilution of 1:1000, washed again, incubated with horseradish peroxidase (HRP)-linked Fc region-specific goat anti-human IgG secondary antibody (Ab) (Southern Biotech, Birmingham, AL, USA) and exposed to o-phenylenediamine dihydrochloride (OPD) substrate. The absorbance (450 nm) of each sample was measured in a Labsystems Multiskan Plus (Fisher Scientific) microtiter plate reader (Table 1 and Supplementary file 1). Washing between incubations was done with PBS-T buffer. Standard positive and negative control sera were included on each plate.

Immunoblot assays

Immunoblots were performed following standard procedures.¹³ Briefly, TIB-153 cells grown in RPMI-1640 supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 20 U/ml penicillin and 20 µg/ml streptomycin were washed with PBS and lysed in NP-40 lysis buffer containing 1 µg/ml pepstatin A, 2 µg/ml antipain, 2 µg/ml chymostatin, 2.5 µg/ml leupeptin, and 1 mM Pefabloc SC (1×10^8 cells per 1 ml lysis buffer). The total protein concentration in the cell lysates was estimated using the DC Protein Assay Kit (Bio-Rad) and 37.5 µg of total protein was loaded per well. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes.

Membranes were blocked with 3% BSA, incubated in the presence of 1:10,000 dilutions of test and control antisera, incubated with HRP-linked Fc region-specific goat anti-human IgG secondary Ab (Southern Biotech, Birmingham, AL, USA) and visualized with chemiluminescence (Pierce, Rockford, IL, USA) (Figure 1). The number of positive samples, as determined by the commercial ELISAs, the non-commercial ELISAs and immunoblots (U1-70K, U1A, SmB and SmD) (Table 1), were compared using the two-tailed Fisher's exact test in GraphPad Software, where the *p*-value is calculated using the method of summing small *p*-values.

Determining total IgG concentration in sample sera by direct ELISA

Direct ELISAs were utilized to assess the relative levels of IgG across the sera samples. A standard curve was constructed using purified human IgG (Thermo Scientific, Rockford, IL, USA) in serial dilutions (100 µg/ml to 0.16 µg/ml) in PBS with 10 mg/ml BSA (Supplementary file 2). Control IgG dilutions and diluted sample sera (pre-dissolved in 0.05% BSA/PBS at a 1:100 dilution) were linked to maxisorp flat bottom 96-well ELISA plates (Nunc, Rochester, NY, USA) overnight at 4°C. Plates were washed two times each with 200 µl of PBS-T prior to blocking for 2 h at room temperature with 10 mg/ml BSA in PBS. Subsequently, the wells were incubated with a final concentration of 0.2 µg/ml horseradish peroxidase conjugated anti-human IgG secondary antibody (Southern Biotech, Birmingham, AL, USA) in PBS-T for 1 h at room temperature. Wells were washed four times as previously mentioned and exposed to OPD substrate for 30 min at room temperature. Reactions were stopped by addition of 3M HCl and absorbances were read at 490nm on an ELx808 Absorbance Ultra Microplate Reader (Supplementary file 3). All assays were performed in triplicate. Serum and conjugate controls were included in this assay.

Peptide selection and synthesis

Amino acid sequences from the human U1 snRNP proteins U1A, U1-70K, U1C, SmB, SmD1, SmD2, SmD3, SmE, SmF and SmG were downloaded from the National Center for Biotechnology Information Protein Database and input into the B-cell Epitope Prediction (BepiPred) 1.0 Server (<http://www.cbs.dtu.dk/services/BepiPred/>).¹⁵ Using BepiPred 1.0, a total of 15 peptides were selected to cover all U1 snRNP proteins and a wide range of predicted antigenicities (arbitrarily labeled 1–15) (Table 2). Two additional peptides (16 and 17; Table 2), selected at random, were also utilized as control, in which the primary amino acid sequences had been scrambled. To generate the scrambled sequences, peptides 16 and 17 were re-sampled at random from the sequences of peptides 5 and 10, respectively, using a random number generator from 1 to 8. All peptides described above were commercially synthesized by BioMatik Corporation (Wilmington, DE, USA) to greater than 90% purity, as determined by high performance liquid chromatography (HPLC).

Monitoring peptide antigenicity by indirect ELISA

Peptides were dissolved in PBS with 10 mg/ml BSA and 0.5mM Phenylmethanesulfonyl Fluoride (PSMF). The antigenicity of each peptide was determined using indirect ELISAs. ELISAs using small peptides have previously been used to assess the antigenicity of U1

snRNP proteins (see James and Harley for a representative study).¹⁶ Briefly, peptides were linked, washed and blocked as previously mentioned above. Subsequently, plates were incubated in the presence of a 1:100 dilution of patient or control serum for 1 h at room temperature. The wells were then washed and incubated in the presence of HRP-conjugated anti-human IgG secondary antibody (at a final concentration of 0.2 µg/ml). Wells were exposed to OPD substrate, reactions were stopped with 3M HCl and absorbances were measured at 490nm on an ELx808 Absorbance Ultra Microplate Reader (Supplementary files 4 and 5). All assays were performed in triplicate. Negative controls, in which BSA/PBS-T took the place of serum, secondary antibody or substrate, were included for each peptide tested. Serial dilutions of antisera ranging from 1:50 to 1:3200 were also used in ELISAs, as described above, to assure that a 1:100 dilution of SLE, MCTD and healthy sera was in the linear range of the indirect ELISA. Positivity cut-off values for each of the peptides included in this study (Table 2) were determined employing the upper tail of the *t*-distribution of the healthy sera readings to attain the upper prediction limit (Table 3 and Supplementary file 6). This method was statistically defined by Frey et al.,¹⁷ which is calculated by the following equation:¹⁸

$$\text{Cut off} = X + SDt\sqrt{(1+1/n)} \quad (1)$$

where *X* is the mean of the healthy sera absorbance readings, *SD* is the standard deviation, *n* is the number of healthy samples and *t* is the one-tailed *t*-distribution using one degree of freedom.

A paired-sample *t*-test was employed using PASW (version 18) to evaluate differences between mean antigenicity of scrambled versus specific peptides at the 95% confidence interval (CI). ANOVA was performed using PASW to determine whether there were significant differences between the reactivity of the three groups (SLE, MCTD and healthy). Any *p*-value <0.05 was considered significant. We also used one-way ANOVA and Tukey's post-hoc correction to evaluate differences in peptide reactivity between the different ethnic groups (African American and Latin American). We analyzed the relationship between age and peptide reactivity first using a simple linear regression and also by separating the patients into three age groups (19–35, 36–49 and 50 or older) and evaluating differences in age group using a one-way ANOVA. Any *p*-value <0.05 after Tukey's post-hoc correction was considered statistically significant. Receiver operating characteristic (ROC) curves were generated in PASW (Figure 2). The antigenicities of the two peptides with the highest diagnostic power to discriminate between ill and healthy individuals as well as the two autoimmune disorders, as determined by ROC curves, were related to clinical symptoms using a one-way ANOVA with a Tukey's post-hoc test (*p* < 0.05) in PASW using a given clinical symptom as a grouping variable.

The ability of the peptides to segregate the three sample groups (SLE, MCTD and healthy) was determined using principal component analysis (PCA) (Figure 3) in NTSYSpc (version 2.2). PCA is a multivariate statistical technique that transforms the data to capture the greatest amount of variance in the first coordinate series, known as the first principal component. The statistic then transforms the data to capture the next highest amount of

variance in the next principal component, and so on.¹⁹ This results in reduction of noise and clustering of data points that share similar properties.¹⁹

U1 snRNP crystal structure and modeling

The crystal structure of the U1 snRNP was downloaded from the Protein Data Bank (PDB) (PDB ID: **3CW1**).⁴ To generate a more complete model of the U1 snRNP, regions that are missing from the provided crystal structure were modeled and super-imposed onto the empirically-derived U1 snRNP. These segments include the C-terminus of U1C, U1 snRNA stem-loop II and the U1A protein. The three-dimensional model of the U1 snRNA utilized here was developed by Dr Eric Westhof and is freely available online (<http://www-ibmc.u-strasbg.fr/upr9002/westhof/>). The U1A and U1C proteins were modeled using the I-TASSER server (<http://zhang.bioinformatics.ku.edu/I-TASSER/>). The U1A protein was docked to stem-loop II using the PATCHDOCK server (<http://bioinfo3d.cs.tau.ac.il/PatchDock/>) based on the known interactions between U1 snRNA and U1A.²⁰ The solution with the closest match to the empirically derived interaction²⁰ was selected for superposition with the U1 snRNP crystal structure. Following docking of U1A to stem-loop II, the double stranded portion of stem-loop II was superimposed over the kissing loop fragment corresponding to stem-loop II in the crystal structure⁴ using the MatchMaker tool in UCSF Chimera (version 1.3). The U1C top model was then superimposed onto the N-terminal fragment of U1C in the crystal structure using UCSF Chimera. The C-terminus of U1-70K (amino acids 181–437) is also missing from the crystal structure of the U1 snRNP in the PDB; however, the top model of U1-70K from I-TASSER comprises largely low complexity regions which present difficulty in assigning the C-terminal region of U1-70K to a particular area in three-dimensional space. As a result, the missing fragment of U1-70K was not included in the antigenicity map of the U1 snRNP.

Generation of U1 snRNP epitope maps

Two epitope maps of the U1 snRNP complex were generated (Figures 4 and 5). The first map (Figure 4) employed peptides that were gathered from the literature and shown in previous investigations to elicit an autoimmune response in patients with SLE (Table 4).^{16,21–27} The epitope map was created by rendering these previously investigated antigenic U1 snRNP epitopes onto the U1 snRNP crystal structure in red using PyMOL (Figure 4).

A second epitope map (Figure 5) was also generated using the peptides tested in the present study. The peptides tested in the present work were utilized to develop a heat map of reactivity on the U1 snRNP using the Colorama plug-in for PyMOL (Figure 5). First, the peptides studied in the present work were ranked 1 to 15 according to their average antigenicity among SLE patients. Peptides were then given a percentile score relative to their rank. The heat map is on a color scale from blue to red, with blue being the least antigenic of the peptides tested, white in the middle and red the most antigenic (Figure 5). Peptides ranked from the 0 to 50th percentile are blue to white while those that are in the 51st to 100th percentile are white to red. Peptides were assigned the appropriate series of values in the RGB color scheme for each of the three colors based on their relative percentile rank and plotted onto the U1 snRNP model using the Colorama plug-in for PyMOL (Figure 5).

Results

To investigate the relative autoantigenicity of specific segments of the U1 snRNP in individuals with SLE and MCTD, a series of 15 peptides were synthesized and tested with ELISAs using a cohort of 69 SLE and 29 MCTD patients. All patients had been clinically diagnosed with either SLE or MCTD prior to the current study following the American College of Rheumatology (ACR) criteria for the clinical classification of lupus and the Alarcon-Segovia criteria for the clinical classification of MCTD.¹⁴ Two additional peptides (peptides 16 and 17) were also developed as controls in which the amino acid sequence of a peptide from the U1 snRNP had been rearranged at random (highlighted in gray in Table 2). Peptide 16 is a scrambled version of peptide 5 from U1-70K while peptide 17 was derived from peptide 10 from the U1A protein (Table 2). The average autoimmune reactivity of peptides 5 and 10 was significantly greater than those of scrambled peptides 16 and 17, respectively, in patients with SLE ($p=0.001$ and 0.01 , respectively). Similarly, the reactivity of peptide 10 was significantly higher than scrambled peptide 17 in individuals with MCTD ($p=0.001$). In contrast, the reactivity of peptide 5 was not significantly different from that of scrambled peptide 16 in individuals with MCTD ($p=0.090$).

Patient and healthy sera were also tested for the presence of U1 snRNP-specific antibodies using commercial ELISAs (performed by Quest Diagnostics Incorporated), a non-commercial ELISA with purified U1-70K antigen and immunoblots to detect U1-70K, U1A, SmB/B' and SmD proteins (Table 1). We used ANOVA to determine whether there was any relationship between the results of these assays and peptide reactivity. Importantly, we found that the antigenicity of some of the peptides is significantly correlated with the results from commercial and noncommercial ELISAs and western blots ($p < 0.05$). For example, there is a significant relationship between individuals who have the highest reactivity to the U1-70K peptides (5 and 14) and those who were positive in the non-commercial U1-70K ELISA ($p=0.005$ and 0.002 for peptides 5 and 14, respectively) as well as the U1-70K Western blot ($p=0.023$ for peptide 5 in SLE patients alone, and $p=0.045$ for peptide 14 in SLE and MCTD patients). These relationships were also observed for SmB ($p=0.003$ when comparing peptide 6 to the non-commercial SmB/B' Western blot). The same comparisons could not be made with the commercial RNP ELISA due to the high number of RNP-positive samples in the dataset (79.3% of SLE and 100% of MCTD patients).

Total IgG concentration in sample sera does not correlate with reactivity to U1 snRNP peptides

The IgG concentration in each sample was assessed by direct ELISA (Supplementary files 2 and 3). Considering that the samples from SLE and MCTD patients have higher levels of total IgG, it is possible that the increased response to the U1 snRNP peptides is simply due to the increased IgG in the serum. Purified human IgG (Invitrogen) exhibits a strong linear relationship with peptide reactivity ($R^2=0.9783$) (Supplementary file 7). Similarly, healthy individuals also display a moderately strong relationship between total IgG concentration and peptide reactivity ($R^2=0.5386$) (Supplementary file 7). Yet, in stark contrast to the non-specific, purified IgG and the samples from healthy volunteers, there is a very weak correlation between total IgG concentration and reactivity to the U1 snRNP peptides in SLE

and MCTD samples ($R^2=0.1867$ and $R^2=0.2013$, respectively) (Supplementary file 7), which suggests that the significantly higher response to the peptides in the SLE and MCTD groups is not the result of increased total IgG in the serum.

Individuals from different demographic groups exhibit variability in their responses to U1 snRNP autoantigens

Previous research indicates that autoantigenicity is higher in certain demographic groups, including women²⁸ as well as the African American and Hispanic/Latin American populations.²⁹ Interestingly, in this study 98% of the SLE and MCTD sub-groups are women. The cohorts were also separated into two sub-populations based on ethnicity (African and Latin Americans) to determine whether these sub-divisions display significantly different reactivity to the 15 peptides (Figure 6). As expected, individuals (SLE, MCTD and healthy) who classified themselves as African American exhibited significantly higher antigenicity to the U1 snRNP peptides than those who categorized themselves as Latin American ($p < 0.0001$). In addition, the reactivity of almost all the peptides was significantly higher in individuals who called themselves African American compared with those who thought of themselves as Latin American (Figure 6). We also investigated whether there was any relationship between age and peptide antigenicity as measured by indirect ELISA. To do this, we used a simple linear regression to observe whether age correlated with antigenicity. We also divided the samples into three age groups (19–35, 36–49, and 50 or older). The youngest age group (19–35) exhibited significantly higher average peptide reactivity ($p < 0.05$) compared to both the other age sub-divisions in the healthy and MCTD populations. In the SLE patients, the youngest age group displayed significantly higher average peptide reactivity ($p=0.004$) than the oldest age group (50 years old) (data not shown).

An epitope map of the U1 snRNP

Epitope maps generated with previously reported peptides (Table 4), as well as the 15 peptides assayed here, reveal autoantigens of varying structure and position in the U1 snRNP complex (Figures 4 and 5, respectively). Interestingly, there is no apparent bias for epitopes to conform to a particular structure, with loops, β -strands and sheets and α -helices all targeted by autoantibodies; however, many of the segments of SmB, D1, D2 and D3 that share similar folds are antigenic.

As expected, most of the U1 snRNP antigenic sites reside on the surface of the complex (Figure 4). For example, the alpha helical segment of U1-70K (residues 63–84) that passes along the edge of stem-loop I, the C-terminal helix of U1C (residues 57–60) and the N-terminal (residues 30–41) and C-terminal (residues 111–114) loops of SmD2 are highly exposed (boxes in Figure 4). Conversely, several epitopes may be accessible as monomers, but appear unreachable by autoantibodies in the context of the U1 snRNP complex (Figure 4). For example, the RNA binding domains of U1A (residues 35–59) and U1-70K (residues 139–151) become buried upon binding to U1 snRNA (indicated by arrows in Figure 4), but remain antigenic. Three additional antigenic sites within the loop formed by the Sm core

through which the stem of U1 snRNA stem-loop IV passes are also exposed as monomers, but inaccessible when bound to U1 snRNA.

The average antigenicity for each of the 15 peptides tested here was mapped onto the U1 snRNP and a second heat map was generated (Figure 5). Peptides 5 and 14 (amino acids 337 to 344 and 325 to 332 from the U1-70K, respectively) are missing from the recently generated U1 snRNP crystal structure.⁴ As a result, these peptides could not be placed within the context of the U1 snRNP; yet, they were modeled using I-TASSER and the conformation and antigenicity of both peptides are shown in the box at the bottom of Figure 5. The two most antigenic peptides are from amino acids 66 to 73 and 90 to 97 of the U1C protein while the least antigenic is from residues 178–185 of the U1A protein (Table 2). Surprisingly, 11 out of 15 peptides ranked below the 50th percentile of relative antigenicity, with just four peptides (peptide 4 from U1C, peptide 5 from U1-70K, peptide 6 from SmB and peptide 15 from U1C) above the 50th percentile relative to peptide 15 from the U1C protein (Table 2). These data suggest that while most of the peptides tested from the U1 snRNP are mildly to moderately antigenic, a subset of sites on the complex elicit a very strong autoimmune response in patients with SLE and MCTD (Figure 5).

Most of the sites that were selected by the BepiPred 1.0 server are on the surface of the U1 snRNP crystal structure, with the exception of peptides 7, 8, 12 and 15 (SLE observed antigenic ranking of 12, 5, 15 and 1, respectively, in Table 2), which appear to be more buried in the snRNP complex (Figure 5). As indicated in the epitope map that was generated using previously published peptides (Figure 4), the antigenicity of a subset of peptides is not solely dependent on its overall accessibility in the context of either proteins as monomers or the entire U1 snRNP. A striking example from the current dataset is peptide 9 from SmF, which is perhaps the most exposed and accessible epitope of the peptides tested in the present study (Figure 5). This peptide ranks fifth in observed antigenicity for SLE among the 17 peptides (15 U1 snRNP peptides and two scrambled) (Table 2). Furthermore, peptides 4 and 15 from U1C are among the most antigenic for both SLE and MCTD, but appear to be more buried within the U1 snRNP (Figure 5). Although our modeled region of U1C places peptide 15 internally in the context of the U1 snRNP, the crystal structure positions the amino acids directly to the N-terminus of this protein away from the complex, which would greatly increase the accessibility of this peptide.

U1 snRNP peptides as diagnostic markers for SLE and MCTD

Both SLE and MCTD patient populations demonstrate highly variable reactivity to the peptides derived from U1 snRNP proteins. A PCA reveals that while there are typically much higher responses from SLE patients, moderate titers from individuals with MCTD and lower reactivity from healthy volunteers, the wide ranges observed for each group allow for considerable overlap (Figure 3). Generally, the reactivity of the MCTD population falls between the SLE and healthy groups, making it difficult to distinguish the three populations. Although the peptides as a group are able to separate the SLE, MCTD and healthy populations (see the discussion on ROC curves below), the discrimination becomes much less clear at the level of the individual. Considering the significant relationship between ethnicity and autoantigenicity, we also postulated that ethnicity would not effectively

segregate the three groups (SLE, MCTD and healthy) in a PCA. As expected, PCAs performed using ethnicity as a grouping variable did not effectively separate the samples into distinct groups (data not shown).

In an effort to further understand which peptides may be the most useful in delineating the three clinical sub-divisions, ROC curves were constructed for each peptide (Figure 2). ROC curves represent a graphical plot of the relationship between the sensitivity and specificity of a given diagnostic marker (in this case, each of the peptides). A perfect prediction would correspond to a point in the upper left corner of the graph, representing the coordinates 0, 1, which indicates perfect sensitivity (no false negatives) and specificity (no false positives). The area under the ROC curve, represented as a number from 0 to 1, indicates the probability that a peptide will correctly predict a randomly chosen individual with SLE or MCTD as positive for the illness compared with a randomly chosen healthy individual. As illustrated in Figure 2, all but two of the peptides (P2 and P9) are able to significantly distinguish SLE and MCTD samples from healthy samples ($p < 0.05$); however, the peptides are unable to significantly separate individuals with MCTD from those with SLE (Figure 2C). Peptide 5 (U1-70K) is the best at predicting which samples are in the SLE group from healthy samples and the second best at separating MCTD from healthy samples ($p=0.0001$). Peptides 2 (SmE) and 9 (SmF) were the worst predictors of the autoimmune diseases (Figure 2, A and B, respectively) and ranked 13th and 17th, respectively, among SLE patients and 5th and 13th, respectively, among MCTD patients (Table 2). While all of the peptides are significantly strong predictors of autoimmune illness with the exception of peptides 2 and 9 for MCTD, none are capable of significantly segregating SLE from MCTD ($p < 0.05$) (Figure 2C). Interestingly, however, peptides 1 and 13, both from the U1A protein, are the two best predictors of SLE vs. MCTD ($p=0.167$ and $p=0.206$, respectively) (Figure 2C). Although the ROC curves illustrate the weak capacity of any of the peptides to discriminate between the two disease states (Figure 2C), the current study indicates that U1A may be the most likely U1 snRNP protein to contain one or more sites that will enable the two diseases to be significantly distinguished.

Correlation of peptides 4 and 5 with clinical symptoms in both groups of patients

As determined by the ROC curves, the two peptides with the greatest statistical ability to properly distinguish individuals with one of the two autoimmune diseases from healthy volunteers were peptides 4 (U1C) and 5 (U1-70K) (Figure 2, A and B, respectively). These peptides were tested for correlations with all of the observed clinical symptoms in both patient groups using a one way ANOVA and corrected by Tukey's post-hoc test ($p < 0.05$). Only samples in which both antigenicity and clinical data were available were used in the comparisons (data not shown). Surprisingly, none of the clinical symptoms were statistically correlated with reactivity to the peptides. It is possible that the relatively low sample sizes (less than 20 per group in some cases) compromised the robustness of the statistical analyses.

Discussion

The rheumatic autoimmune diseases are both highly complex and extremely dynamic. Over 100 SLE autoantigens have been described in the literature, and a wide variation in these antigens exists among individuals.³⁰ With this in mind, we used a series of commercial and non-commercial ELISAs and western blots to characterize the anti-U1 snRNP protein antibodies within sera from patients with SLE and MCTD and healthy volunteers. The results from the commercial ELISAs and the non-commercial ELISAs as well as western blots significantly correlated with patient reactivity against several U1 snRNP peptides ($p < 0.05$). The lack of correlation between the western blots and the peptide ELISAs to detect SmD is not surprising considering the low number of patients ($n=14$) who were positive for SmD in the immunoblots (see Table 1) and the relatively low average peptide reactivity in the indirect ELISAs (out of the 17 peptides tested, SmD peptides rank 5th, 15th and 16th among those with SLE, and 3rd, 11th and 12th among patients with MCTD) (Table 2). Overall, the strong relationships between patient reactivity to the peptides and the results from commercial ELISAs and non-commercial ELISAs and western blots further indicates that the peptide ELISAs can be employed in epitope mapping of the U1 snRNP, which is discussed in greater detail below.

The current study also reveals that there is variation not only among individuals, but within the same individual at different times and during the progression of the disease. For example, there is a 6.36-fold difference in autoantigenicity among all SLE patients in this study. Furthermore, autoimmune reactivity within a single individual with SLE can fluctuate up to 1.81-fold between patient visits ($p=0.001$ using a paired *t*-test). The PCA provides a diagrammatic view of the degree to which peptide reactivity varies among individuals within the three groups (SLE, MCTD and healthy) (Figure 3). It is interesting to note that most of the healthy samples cluster below the SLE and MCTD samples, which suggests that the peptides may be able to segregate the individuals with autoimmune disorders from healthy volunteers. This PCA distribution is also supported by the ROC curves (Figure 2). The ROC curves generated with each of the U1 snRNP peptides show that almost all of the peptides are able to significantly partition the healthy individuals from those with SLE or MCTD and with high probabilities. The ROC curves also confirm that the peptides are not able to significantly distinguish between the SLE and MCTD groups (Figure 2C). Taken together, these analyses indicate that the wide range of autoimmune reactivity within a given population, coupled with the significant overlap among the three clinical sub-groups (SLE, MCTD and healthy) (Figure 3), make diagnoses based on just one or two clinical characteristics difficult.

A number of previous investigations have demonstrated connections between the prevalence and/or severity of autoimmune disorders with different demographic groups (please refer to Pons-Estel et al. for a review of the literature).³¹ Specifically, in the United States, women of African American and other minority backgrounds have a higher incidence of SLE.³¹ Similarly, the SLE and MCTD cohort studied here was comprised of over 98% women. Disease severity is also higher in 'ethnic minority' groups.³¹ Likewise, the current results indicate that those individuals who classified themselves as African American had significantly higher average reactivity to the U1 snRNP peptides than those who categorized

themselves as Latin American (Figure 6). Unfortunately, there were not enough individuals in this cohort who thought of themselves as Caucasian for us to observe differences between this group and those that would typically be classified as 'ethnic minority' groups. Yet, our data further support the notion that African American women have a higher incidence and greater severity of SLE and MCTD than other groups. We also found a significant relationship between peptide reactivity and age, with higher reactivity to the peptides in younger individuals (19–35 years old) in both the SLE and MCTD populations ($p=0.05$).

The development of U1 snRNP epitope maps reveals that most autoantigenic peptides represent accessible regions on the surface of the U1 snRNP (Figure 4). The idea that autoantibodies target surface structures has been well established in the literature. For example, McClain et al.²⁷ found that antibodies against the Sm proteins were directed largely against basic epitopes on the outer surfaces of the polypeptides. Later, this same group reported that exposed areas of the U1A protein were antigenic.²⁴ In the epitope maps generated here, helices of U1-70K and U1C and loop regions of the Sm complex (boxed in Figure 4) are among the previously described antigenic sites that are highly accessible on the U1 snRNP assembly.

Perhaps more interesting is that some exposed structural elements on the U1 snRNP are not antigenic (Figure 5). In particular, the N-terminal region of U1-70K that contacts SmB and SmD2 is particularly exposed, but has not yet been identified as an autoantigenic site. Amino acids within SmB and SmD2 that contact U1-70K are also not antigenic, nor are areas of contact between Sm proteins, with the exception of a portion of SmD1 (residues 66–76) that contacts the SmD2 protein. The observation by McClain and colleagues²⁷ that antigenic regions within Sm proteins are distinctly segregated from residues necessary for protein–protein interaction is recapitulated and further supported in the U1 snRNP crystal structure.

The epitope maps also indicate that autoantibodies may recognize subcomplexes of the U1 snRNP. For example, autoantibodies have been detected in some SLE patients that are directed against conformational epitopes of the U1C-Sm complex.³² These antibodies are thought to stabilize the U1C-Sm interaction and may inhibit proteolysis and subsequent presentation of Sm epitopes to B cells.³² Similarly, patients with high anti-Sm titers recognize Sm E-F-G trimeric complexes.¹¹ Furthermore, many of the sites necessary for protein–protein interactions within the U1 snRNP, such as the ones involving U1-70K and SmB and D2 as well as contact points between the Sm proteins, are not antigenic. The finding that autoantibodies against protein-binding amino acids are not abundant was first reported by McClain et al.²⁷ in the context of Sm dimers. Our results support the findings that autoantibodies may target U1 snRNP subcomplexes, with most antigenic sites positioned away from areas of protein–protein association (Figure 5), likely the result of inaccessibility as a potential antigen.

While there is a tendency for autoantibodies to target residues involved in protein–protein interactions, the epitope maps generated in the present work indicate that this is not the case with sites that are necessary for protein–RNA associations. For example, the RNA binding domains of U1-70K and U1A, as well as the regions of several Sm proteins that contact the

stem of stem-loop IV, are all antigenic targets (Figure 4). In fact, some have suggested that U1 snRNA may contribute to the antigenicity of U1-70K through innate immunity signaling.³³ These, and other sites, may also become accessible upon apoptosis-induced cleavage by caspases or other proteases. In fact, there is considerable evidence that increased proteolytic cleavage and other apoptotic modifications of U1 snRNP proteins may create novel autoantigens.^{9,11,13,34} For example, the RNA binding domain of U1-70K becomes exposed and elicits a strong immune response following apoptotic cleavage.¹³ This may explain why the inaccessible RNA binding domain of U1-70K is so antigenic despite its internal position in the context of the U1 snRNP.

It is noteworthy that peptide 4 from U1C elicits the strongest response in both SLE and MCTD patients of the group of 15 U1 snRNP peptides tested (Figure 5). To our knowledge, there is no precedent for higher reactivity against U1C epitopes than U1-70K or the Sm proteins in both disease types; however, this peptide is highly pro-line-rich, a characteristic that is common to previously analyzed autoantigenic epitopes.^{25,35,36} Hoet and colleagues³⁷ also found two U1C epitopes using combinatorial human antibody libraries. The antigenic sites were localized to amino acids 30–63 and 98–126 of U1C.³² These regions are just a few amino acids away from each of the two highly antigenic U1C peptides tested here (Table 2).

It is not surprising that peptides from the U1-70K protein are among the most antigenic of the 15 U1 snRNP peptides tested in the present investigation (Figure 5 and Supplementary file 5). Autoantibodies against the U1-70K molecule are observed early in the onset of rheumatic diseases including SLE and MCTD, and are known to contribute to progression of these illnesses through epitope spreading.^{25,38,39} Our findings support these previous investigations and indicate that the presence of autoantibodies directed at U1-70K may be useful in diagnosing whether an individual has either of these two autoimmune inflammatory illnesses.

The current study highlights the complex nature of autoantigens on the U1 snRNP. While many U1 snRNP epitopes represent surface structures, there also appear to be additional elements that contribute to the autoantigenicity of a given protein fragment, including conformational epitopes made up of multiple proteins, autoantigens derived from RNA-protein complexes and/or epitopes formed by way of apoptosis-induced proteolytic digestion. In addition to the characteristic intricacy observed among autoantigens, a wide range of antigenicity also exists among patients and even within individuals at different times. Furthermore, the substantial overlap in antigenicity between the SLE and MCTD groups precludes the ability of any peptides used in the present study to effectively partition the two disease states. Taken together, these data underscore the multiplicity of dynamic and often confounding qualities inherent in autoimmune illnesses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Pettersson I, Hinterberger M, Mimori T, Gottlieb E, Steitz JA. The structure of mammalian small nuclear ribonucleoproteins. Identification of multiple protein components reactive with anti-(U1)ribonucleoprotein and anti-Sm autoantibodies. *J Biol Chem.* 1984; 259:5907–5914. [PubMed: 6232278]
- Combe B, Rucheton M, Graafland H, Lussiez V, Brunel C, Sany J. Clinical significance of anti-RNP and anti-Sm autoantibodies as determined by immunoblotting and immunoprecipitation in sera from patients with connective tissue diseases. *Clin Exp Immunol.* 1989; 75:18–24. [PubMed: 2702772]
- Riemekasten G, Hahn BH. Key autoantigens in SLE. *Rheumatology.* 2005; 44:975–982. [PubMed: 15901907]
- Pomeranz Krummel DA, Oubridge C, Leung AKW, Li J, Nagai K. Crystal structure of human spliceosomal U1 snRNP at 5.5 Å resolution. *Nature.* 2009; 458:475–480. [PubMed: 19325628]
- Luyckx A, Westhovens R, Oris E, Papisch W, Bossuyt X. Clinical relevance of measurement of antibodies to individual snU1-RNP proteins. *Clin Chem.* 2005; 51:1888–1890. [PubMed: 16099934]
- Margaux J, Hayem G, Palazzo E, et al. Clinical usefulness of antibodies to U1snRNP proteins in mixed connective tissue disease and systemic lupus erythematosus. *Rev Rhum (English ed.).* 1998; 65:378–386.
- Keith MP, Moratz C, Tsokos GC. Anti-RNP immunity: implications for tissue injury and the pathogenesis of connective tissue disease. *Autoimmun Rev.* 2007; 6:232–236. [PubMed: 17317614]
- Malmegrim KCR, Pruijn GJM, van Venrooij WJ. The fate of the U1 snRNP autoantigen during apoptosis: Implications for systemic autoimmunity. *Isr Med Assoc J.* 2002; 4:706–712. [PubMed: 12440236]
- Hof D, Raats JMH, Pruijn GJM. Apoptotic modifications affect the autoreactivity of the U1 snRNP autoantigen. *Autoimmun Rev.* 2005; 4:380–388. [PubMed: 16081029]
- Janko C, Schorn C, Grossmayer GE, et al. Inflammatory clearance of apoptotic remnants in systemic lupus erythematosus (SLE). *Autoimmun Rev.* 2008; 8:9–12. [PubMed: 18703173]
- Malmegrim de Farias KCR, Saelens X, Pruijn GJ, Vandenabeele P, van Venrooij WJ. Caspase-mediated cleavage of the U snRNP-associated Sm-F protein during apoptosis. *Cell Death Differ.* 2003; 10:570–579. [PubMed: 12728255]
- Greidinger EL, Foecking MF, Ranatunga S, Hoffman RW. Apoptotic U1-70 kd is antigenically distinct from the intact form of the U1-70-kd molecule. *Arthritis Rheum.* 2002; 46:1264–1269. [PubMed: 12115232]
- Greidinger EL, Foecking MF, Magee J, Wilson L, Ranatunga S, Ortmann RA, Hoffman RW. A major B cell epitope present on the apoptotic but not the intact form of the U1-70-kDa ribonucleoprotein autoantigen. *J Immunol.* 2004; 172:709–716. [PubMed: 14688384]
- Maldonado ME, Perez M, Pignac-Kobinger J, et al. clinical and immunological manifestations of mixed connective tissue disease in a Miami population compared to a Midwestern US Caucasian population. *J Rheumatol.* 2008; 35:429–437. [PubMed: 18260175]
- Larsen JEP, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. *Immunome Res.* 2006; 2:2. [PubMed: 16635264]

16. James JA, Harley JB. Human lupus anti-spliceosome A protein autoantibodies bind contiguous surface structures and segregate into two sequential epitope binding patterns. *J Immunol.* 1996; 156:4018–4026. [PubMed: 8621944]
17. Frey A, Di Canzio J, Zurakowski D. A statistically defined endpoint titer determination method for immunoassays. *J Immunol Methods.* 1998; 221:34–41.
18. Dixon, WJ.; Massey, FJ. *Introduction to Statistical Analysis*, 4th edn. New York: McGraw-Hill; 1983. p. 92
19. Ramenofsky AF, Neiman F, Pierce CD. Measuring time, population and residential mobility from the surface at San Marcos pueblo, North Central, New Mexico. *Am Antiq.* 2009; 74:1–26.
20. Oubridge C, Ito N, Evans PR, Teo C-H, Nagai K. Crystal structure at 1.92 Å resolution of the RNA-binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. *Nature.* 1994; 372:432–438. [PubMed: 7984237]
21. Arbuckle MR, Schilling AR, Harley JB, James JA. A limited lupus anti-spliceosomal response targets a cross-reactive, proline-rich motif. *J Autoimmun.* 1998; 11:431–438. [PubMed: 9802926]
22. Talken BL, Schafermeyer KR, Bailey CW, Lee DR, Hoffman RW. T cell epitope mapping of the Smith antigen reveals that highly conserved Smith antigen motifs are the dominant target of T cell immunity in systemic lupus erythematosus. *J Immunol.* 2001; 167:562–568. [PubMed: 11418695]
23. Barakat S, Briand JP, Abuaf N, Van Regenmortel MHV, Muller S. Mapping of epitopes on U1 snRNP polypeptide A with synthetic peptides and autoimmune sera. *Clin Exp Immunol.* 1991; 86:71–78. [PubMed: 1717192]
24. McClain MT, Lut CS, Kaufman KM, Faig OZ, Gross TF, James JA. Structural availability influences the capacity of autoantigenic epitopes to induce a widespread lupus-like autoimmune response. *Proc Nat Acad Sci.* 2004; 101:3551–3556. [PubMed: 14988508]
25. Poole BD, Schneider RI, Guthridge JM, et al. Early targets of nuclear RNP humoral autoimmunity in human systemic lupus erythematosus. *Arthritis Rheum.* 2009; 60:848–859. [PubMed: 19248110]
26. Henriksson EW, Pettersson I. Autoepitope-mapping of the U1-70K protein with human–*Drosophila* chimeric proteins. *J Autoimmun.* 1997; 10:559–568. [PubMed: 9451595]
27. McClain MT, Ramsland PA, Kaufman KM, James JA. Anti-Sm autoantibodies in systemic lupus target highly basic surface structures of complexed spliceosomal autoantigens. *J Immunol.* 2002; 168:2054–2062. [PubMed: 11823543]
28. Petri M. Review for classification criteria for systemic lupus erythematosus. *Rheum Dis Clin North Am.* 2005; 31:245–254. [PubMed: 15922144]
29. Borchers T, Naguwa SM, Shoenfeld Y, Gershwin ME. The epidemiology of systemic lupus erythematosus. *Autoimmun Rev.* 2010; 5:A277–A287. [PubMed: 20036343]
30. Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin Arthritis Rheum.* 2004; 34:501–537. [PubMed: 15505768]
31. Pons-Estel GJ, Alarcón GS, Scofield L, Reinlib L, Cooper GS. Understanding the epidemiology and progression of systemic lupus erythematosus. *Semin Arthritis Rheum.* 2010; 39:257–268. [PubMed: 19136143]
32. Satoh M, Akaogi J, Kuroda Y, et al. Autoantibodies that stabilize U1snRNP are a significant component of human autoantibodies to snRNP and delay proteolysis of sm antigens in vitro. *J Rheumatol.* 2004; 31:2381–2389.
33. Hoffman RW, Gazitt T, Foeking MF, et al. U1 RNA induces innate immunity signaling. *Arthritis Rheum.* 2004; 50:2891–2896. [PubMed: 15457457]
34. Dieker J, Cisterna B, Monneaux F, et al. Apoptosis-linked changes in the phosphorylation status and subcellular localization of the spliceosomal autoantigen U1-70K. *Cell Death Differ.* 2008; 15:793–804. [PubMed: 18202700]
35. Kaufman KM, Kirby MY, Harley B, James JA. Peptide mimics of a major Lupus epitope of SmB/B. *Ann NY Acad Sci.* 2003; 987:215–229. [PubMed: 12727642]
36. Zieve GW, Khusial PR. The anti-Sm immune response in autoimmunity and cell biology. *Autoimmun Rev.* 2003; 2:235–240. [PubMed: 12965173]

37. Hoet RM, Raats JM, de Wildt R, et al. Human monoclonal autoantibody fragments from combinatorial antibody libraries directed to the U1snRNP associated U1C protein; epitope mapping, immunolocalization and V-gene usage. *Mol Immunol.* 1998; 35:1045–1055. 1998. [PubMed: 10068039]
38. Keith MP, Moratz C, Egan R, Zacharia A, Greidinger EL, Hoffman RW, Tsokos GC. Anti-ribonucleoprotein antibodies mediate enhanced lung injury following mesenteric ischemia/reperfusion in Rag-1(–/–) mice. *Autoimmunity.* 2007; 40:208–216. [PubMed: 17453720]
39. Monneaux F, Parietti V, Briand JP, Muller S. Importance of spliceosomal RNP1 motif for intermolecular T-B cell spreading and tolerance restoration in lupus. *Arthritis Res Ther.* 2007; 9:R111. [PubMed: 17963484]

Figure 1.

Screening patient sera for the presence of anti-U1-70K, -U1A, -SmB/B' and -SmD1/D2/D3 antibodies by immunoblotting. A. TIB-153 Jurkat cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated in the presence of 1:10,000 dilutions of primary antisera from U1-70K-positive/Sm-negative control (lane 1), U1-70K-positive/Sm-positive control (lane 2) and healthy control (lane 3). Lanes 4–6 were incubated with increasing dilutions (1:100, 1:1000 and 1:10000, respectively) of anti-Sm monoclonal antibody Y12/SPM318 (Novus Biologicals, Littleton, CO, USA) as controls while patient

test samples are represented in lanes 7–12. B. TIB-153 Jurkat cell lysates electrophoresed by 15% SDS-PAGE, transferred to nitrocellulose membranes and exposed to 1:10,000 dilutions of primary antisera from U1-70K-positive/Sm-negative control (lane 1), U1-70K-positive/Sm-positive control (lane 2), healthy control (lane 3) and patient test samples (lanes 4–12).

Figure 2.

Receiver operating characteristic (ROC) curves delineate the peptides with the most powerful diagnostic power. The peptide with the greatest area under the curve (AUC) (second column in each table, labeled 'area') is the most robust at correctly predicting which sera are from patients with SLE. **A.** All peptides are capable of significantly distinguishing ($p < 0.05$) the SLE group from healthy individuals as determined by the asymptotic significance. Peptide 5 (U1-70K) is the most statistically reliable at predicting the SLE state (AUC=0.922). **B.** With the exception of two (peptides 2 and 9 from SmE and SmF,

respectively), all peptides are able to statistically differentiate between MCTD and healthy sera. Peptides 4 (U1C) and 5 (U1-70K) are the most statistically reliable (AUC=0.907 and 0.910, respectively). **C.** None of the peptides can statistically segregate the samples from patients with SLE from those with MCTD. Although it is not statistically significant, peptide 1 (U1A) is the most reliable at differentiating SLE from MCTD samples (AUC=0.576; $p=0.167$).

Figure 3.

Principal component analysis reveals substantial overlap among SLE, MCTD and healthy sera. All 15 U1 snRNP peptides were utilized in the principal component analysis (PCA) in an effort to distinguish among the three populations (SLE, MCTD and healthy). The PCA shows that although a subset of individuals with SLE (designated L1 through L68 with red dots) are clearly segregated from the healthy group (H1 through H26 with white circles), individuals with MCTD (M1 through M29 with blue dots) cluster with either the SLE or healthy populations. The PCA graphically demonstrates the diversity of antigenic reactivity among individuals within each of the three groups.

Figure 4.

An autoimmune epitope map of the U1 snRNP based on previous studies. Antigenic sites within the U1 snRNP complex were collected from the literature and rendered in red on the U1 snRNP crystal structure (PDB ID: 3CW1) using PyMOL. U1 snRNA stem-loops (I, II, III and IV) and U1 snRNP proteins (U1A, U1C, U1-70K and Sm core) are indicated. While many of the antigens are on the outer surface of the complex (examples are indicated with boxes), some epitopes remain inaccessible in the context of the U1 snRNP. Regions of U1-70K and U1A that bind U1 snRNA are delineated with arrows.

Figure 5.

A heat map of the U1 snRNP reveals a small subset of highly antigenic regions. The relative antigenicity of each peptide was plotted onto the crystal structure of the U1 snRNP and a heat map was generated. Peptides with antigenicities ranging from the 0th to the 50th percentile are colored from blue to white while those in the 51st to 100th percentile are colored from white to red. The main structural subunits of the U1 snRNP (U1A, U1C, U1-70K and the Sm core) are designated. Peptides were assigned arbitrary numbers from 1 to 17. Peptides 5 and 14 correspond to C-terminal fragments of U1-70K, a region that was not included in the U1 snRNP crystal structure. As a result, these peptides are separated from the U1 snRNP in the figure.

Figure 6.

Relationship between peptide reactivity and ethnicity in SLE, MCTD and control sera. The reactivity of each ethnic group (African American or Latin American) to the individual peptides (labeled P1–P15) and the average of all peptides (labeled “Total (all peptides)”) are represented on the Y axis. Black bars correspond to the African American group and gray bars correspond to the Latin American group. Standard error bars are included at the top of each column. The asterisk (*) denotes statistically significant differences ($p < 0.05$) using a one-way ANOVA.

Table 1

Percentage of samples with positive autoantibody results in each diagnosis group

Diagnosis group	Commercial assays		Non-commercial assays			
	RNP*	Sm*	U1-70K* ELISA	U1-70K immunoblot	U1A immunoblot	Smb/B* immunoblot
SLE (N = 82)	84.4 (64/76)	57.1 (43/76)	52.5 (42/80)	55.6 (45/81)	59.3 (48/81)	44.4 (36/81)
MCTD (N = 42)	100.0 (42/42)	24.4 (10/41)	81.0 (34/42)	70.7 (29/41)	70.0 (28/40)	42.5 (17/40)
Healthy (N = 32)	nd (0/0)	nd (0/0)	0.0 (0/2)	0.0 (0/20)	0.0 (0/20)	0.0 (0/20)

Commercial assays were performed by Quest Diagnostics Incorporated.

RNP: anti-U1-ribonucleoprotein antibodies, Sm: anti-Smith antibodies, U1-70K: anti-U1-70K antibodies, U1A: anti-U1A protein antibodies, Smb/B*: anti-Smb/B antibodies, SmD: anti-SmD1/D2/D3 antibodies, N: total number of individuals tested per group, nd: test not done. Fractions in parentheses indicate number of positive samples out of number of samples tested.

* Tests with statistically significant differences when comparing MCTD and SLE results using Fisher's exact test (two-tailed *p*-value < 0.05).

Table 2

U1 snRNP peptides and autoantigenicity

Amino Acid Sequence	U1 snRNP Protein	Amino Acid Position	Peptide Number	Rank Predicted Antigenicity	Rank Observed Antigenicity (SLE)	Rank Observed Antigenicity (MCTD)
APDDGGP	U1-70K	325-332	14	1	10	8
PDGPDGPE	U1-70K	337-344	5	2	4	5
PPDPGGDE	Scrambled	From peptideS	16	3	8	15
EGPPPKDT	Sm B	83-90	6	4	3	4
KPKSQETP	U1A	112-119	10	5	8	6
PFSAPPPA	U1C	66-73	15	6	1	1
EEEEEDGEM	SmF	77-84	9	7	5	13
PTSKKEQP	Scrambled	From peptide 10	17	8	7	10
PPAQPLSE	U1A	196-203	1	5	6	14
WSKAHPPE	Sm G	1-8	7	10	12	9
GQIPPGAM	U1A	173-135	13	11	17	16
CETNTGEV	Sm D3	20-27	8	12	5	3
NTEEYIDG	SmF	46-53	3	13	11	7
EELQKREE	Sm D2	14-21	11	14	16	11
EHSKTKS	Sm E	63-70	2	15	13	17
GMMPAPHM	U1C	90-97	4	16	2	2
GTQVHGTI	Sm D1	22-22	12	17	15	12

Table 3

Percentage of samples with positive U1snRNP autoantibody results in each group

Diagnosis group	Peptide ELISAs														
	P1 ^{*†}	P2	P3 ^{*†}	P4 ^{*†}	P5 ^{*†}	P6 ^{*†}	P7 ^{*†}	P8 ^{*†}	P9 [*]	P10 ^{*†}	P11 ^{*†}	P12 ^{*†}	P13 ^{*†}	P14 ^{*†}	P15 ^{*†}
SLE (N = 82)	52.4% (43/82)	15.9% (13/82)	41.5% (34/82)	68.3% (56/82)	54.9% (45/82)	51.2% (42/82)	36.6% (30/82)	43.9% (36/82)	32.9% (27/82)	48.8% (40/82)	36.6% (30/82)	43.9% (36/82)	20.7% (17/82)	61.0% (50/82)	39.0% (32/82)
MCTD (N = 42)	42.9% (18/42)	19.0% (8/42)	33.3% (14/42)	69.0% (29/42)	47.6% (20/42)	50.0% (21/42)	21.4% (9/42)	42.9% (18/42)	26.2% (11/42)	54.8% (23/42)	33.3% (14/42)	35.7% (15/42)	16.7% (7/42)	45.2% (19/42)	38.1% (16/42)
Healthy (N = 32)	9.4% (3/32)	6.3% (2/32)	6.3% (2/32)	6.3% (2/32)	6.3% (2/32)	6.3% (2/32)	9.4% (3/32)	12.5% (4/32)	6.3% (2/32)	9.4% (3/32)	6.3% (2/32)	9.4% (3/32)	3.1% (1/32)	9.4% (3/32)	6.3% (2/32)

Indirect ELISAs were performed using peptides (P) 1–15. N: total amount of samples tested per group. Fractions in parentheses indicate number of positive samples out of number of samples tested, based on the cut-off values using the upper tail *t* distribution (see Supplementary file 6).

* and † represent statistically significant difference when comparing peptide reactivity from SLE or MCTD, respectively, with that of the control (healthy) group. The statistical analyses were performed using a one-way ANOVA test and Tukey's post-hoc correction test (*p* < 0.032).

Table 4

SLE autoantigens of the U1 snRNP previously detected in other studies

Peptide Sequence	U1 snRNP Protein	Amino Acid Positions	Reference
RSLKMRGQAFVIF	U1A	47–59	Arbuckle et al. ²¹
PPPGMIPP	U1A	165–172	Talken et al. ²²
LVPGRHDIAFVEF	U1A	239–251	Arbuckle et al. ²¹
MAVPETRPNHT	U1A	1–11	Barakat et al. ²³
SQFGQILDILVSRSLKMRGQAFVI	U1A	35–58	Barakat et al. ²³
AGAARDALQGFKITQNNAMKISFAKK	U1A	257–282	Barakat et al. ²³
LVSRSLKMRGQAF	U1A	44–56	McClain et al. ²⁴
ERDRKREKPKPKS	U1A	103–115	McClain et al. ²⁴
KMKGTFVE*	U1A	96–103	Poole et al. ²⁵
TPATKKAVQG*	U1A	118–127	Poole et al. ²⁵
GMPPMTQAPRIM*	U1A	143–154	Poole et al. ²⁵
GQPPYMPPPGMIPPPGLAPG*	U1A	159–178	Poole et al. ²⁵
IPPGAMPPQQLMPG*	U1A	180–193	Poole et al. ²⁵
EVRLVPGR*	U1A	236–242	Poole et al. ²⁵
PAPGMRPP	U1C	118–125	Arbuckle et al. ²¹
FQQGKIPPT*	U1C	57–66	Poole et al. ²⁵
PPPGMMPVGPAPGMRPPMGG*	U1C	109–128	Poole et al. ²⁵
RSGKPRGYAFIEY	U1-70K	139–151	Arbuckle et al. ²¹
GDAFKTLFVARVNYDTTESKLREFEVYGP	U1-70K	99–128	Henricksson and Pettersson ²⁶
MTQFLPPNLL*	U1-70K	1–10	Poole et al. ²⁵
RREERMERKRREKIERRQQEVET*	U1-70K	63–84	Poole et al. ²⁵
PRRLGGGLGGTRRG*	U1-70K	189–202	Poole et al. ²⁵
SRYDERPGPSPLPHRDRD*	U1-70K	217–234	Poole et al. ²⁵
RRERSRERDKERERRRSR*	U1-70K	241–258	Poole et al. ²⁵
KEERRRSRER*	U1-70K	271–280	Poole et al. ²⁵
DGPPGELGPDGP*	U1-70K	329–340	Poole et al. ²⁵
KRDERGSEGRD*	U1-70K	379–390	Poole et al. ²⁵
ENGYLMEAAP*	U1-70K	427–436	Poole et al. ²⁵
PPPGMRPP	SmB/B'	191–198, 216–223, 231–238	Arbuckle et al. ²¹
PPPGMRGP	SmB/B'	223–230	Arbuckle et al. ²¹
PPPGIRGP	SmB/B'	201–208	James and Harley ¹⁶
PGIRGPPP	SmB/B'	203–210	James and Harley ¹⁶
IDYRMRCILQDGRIFGTFAFDKH	SmB/B'	6–30	Talken et al. ²²
RFLMKLSH	SmD1	5–12	James and Harley ¹⁶
NTHLKAVKMTLKNR	SmD1	37–50	James and Harley ¹⁶

Peptide Sequence	U1 snRNP Protein	Amino Acid Positions	Reference
TLKNREPV	SmD1	46–53	James and Harley ¹⁶
ILPDSLPL	SmD1	69–76	James and Harley ¹⁶
DVEPKVKSKKREAVAG	SmD1	82–97	James and Harley ¹⁶
REAVA(GR)10GGPRR	SmD1	92–119	James and Harley ¹⁶
NREPVQLETLSIRGNNIRYFILPD	SmD1	25–49	Talken et al. ²²
VHGTITGVDVSMNTHLKAVKMTLKN	SmD1	49–72	Talken et al. ²²
MSLLNKPSEMP*	SmD2	1–13	McClain et al. ²⁷
SVLTQSVKNNTQ*	SmD2	30–41	McClain et al. ²⁷
WTEVPKSGKKG*	SmD2	74–84	McClain et al. ²⁷
GKKKSKPVNKNDRYISKMFLRGDS*	SmD2	83–105	McClain et al. ²⁷
RNPLIAGK*	SmD2	111–118	McClain et al. ²⁷
TGEVYRGKLIEAD*	SmD3	24–37	McClain et al. ²⁷
QVYIRGSKI*	SmD3	60–68	McClain et al. ²⁷
MLKNAPMLKSMKNKNQGSGAGRGKAAILKAQ*	SmD3	76–106	McClain et al. ²⁷
KAQVAARGRGRGMGRGNIFQKRR*	SmD3	104–126	McClain et al. ²⁷

* Whole protein epitope mapping was done using overlapping peptides, but only those peptides that were previously determined to be the most antigenic are indicated here.