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RAPID COMMUNICATION

Diverse humoral autoimmunity to the ribosomal P proteins in systemic lupus erythematosus and hepatitis C virus infection

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Abstract Autoantibodies to the three ribosomal P proteins (Rib-P) are specifically found in 10% to 40% of systemic lupus erythematosus (SLE) patients. Most anti-Rib-P autoantibodies bind to a C-terminal epitope shared by all three Rib-P proteins P0, P1 and P2. In the present study, we shed more light on the humoral autoimmune response to the Rib-P antigen as it occurs in autoimmunity and infectious disease. In a mutational analysis of the major C-terminal epitope, we verified the key role of phenylalanine residues Phe^{111} and Phe^{114} for binding of most anti-Rib-P serum autoantibodies present in SLE sera (n=28). By nuclear magnetic resonance (NMR) investigation of a peptide comprising the C-terminal 22 amino acids, we observed hallmarks for α-helical secondary structure of the Rib-P epitope core (GFGLFD). Based on NMR data and on SPOT epitope analysis, we propose a structural model of the

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Rib-P major epitope, which displays Phe^{111} and Phe^{114} on one side of the helix. Apart from that, two sera from the hepatitis C virus (HCV) control group (n=68) were found to contain antibodies specific for P2, but not for the other Rib-P proteins. Using a SPOT peptide array scanning the P2 amino acid sequence, we identified reactivity with two distinct epitopes (residues 21–35 and 41–55 of Rib-P2) shared by both HCV sera. We conclude that anti-Rib-P autoreactivity occurs in SLE, Chagas' disease (CD) and—as firstly described here—during HCV infection. Anti-Rib-P reactivity in SLE sera primarily depends on Phe^{111} and Phe^{114} of the α-helical C-terminal epitope. In contrast, anti-Rib-P autoantibodies in HCV infection mainly recognize epitopes within the N-terminal half of ribosomal P2.

Keywords Autoantibodies · SLE · HCV · Ribosomal P · Peptide · Lupus

Introduction

Autoantibodies to the three ribosomal phospho(-P) proteins (Rib-P) are found in 10–40% of patients with systemic lupus erythematosus (SLE) (reviewed in [1]). The target of Rib-P autoantibodies consists of three protein components of the 60S ribosomal subunit designated P0 (38 kD), P1 (19 kD), and P2 (17 kD). A pentameric complex of one copy of P0 and two copies each of P1 and P2 interacts with a 28S rRNA molecule forming a GTPase domain, which is active during the elongation step of protein translation [2].

Anti-Rib-P antibodies have been correlated with the active phase of SLE, lupus nephritis, hepatitis or central nervous system involvement [3–8]. Recent results suggest that there is a direct pathogenic effect of these autoantibodies. For instance, anti-Rib-P antibodies were reported to drive the production of proinflammatory cytokines by peripheral blood monocytes and, just recently, Katzav and colleagues reported the induction of depression in mice by antiribosomal antibodies [9, 10].

The major immunoreactive epitope has been localized to the C terminus, which is common to the three Rib-P proteins [1, 6, 7]. In a recent study, we identified the epitope core (GFGLFD) and suggested amino acids Phe^{111} and Phe^{114} being the key amino acids for antibody binding [7]. Yet, other patient-specific epitopes were found on all three antigens [7, 11–13]. Of high interest, the C terminus of the ribosomal P proteins of *Trypanosoma cruzi* was reported as the antibody target in sera from Chagas' disease (CD) patients [14].

The mechanisms leading to autoimmune syndromes like SLE are not well-understood, however, specific pathogens may contribute to the development of disease. For instance, hepatitis C virus (HCV) infection has been found strikingly associated with autoimmune phenomena including SLE [15–19]. Moreover, it has been reported that cat scratch disease (CSD) can evolve SLE-like symptoms [20]. The latter is of particular interest because the Rib-P C-terminal epitope core shows 100% homology to a stretch of the L-*iso*-aspartyl protein carboxyl methyltransferase homologue (L-APCMH) of *Bartonella henselae*, the pathogen of CSD [7].

In this study, we aim to elucidate the structural composition of the major Rib-P epitope using mutational SPOT peptides and nuclear magnetic resonance (NMR) analysis. We also evaluate cross-reactivity to peptides derived from *Trypanosoma cruzi* and *Bartonella henselae*. Furthermore, we compare anti-Rib-P reactivity in serum samples from SLE patients with those from HCV-infected patients by ELISA and SPOT peptide analysis.

Materials and methods

Serum samples

Sera from patients with autoimmune disorders, such as SLE (n=200), mixed connective tissue disease (MCTD, n=50) and rheumatoid arthritis (RA, n=50), and samples from patients with infectious diseases including HCV (n=68), Epstein Barr virus (EBV, n=20), and cytomegalovirus (CMV, n=20), and from healthy blood donors (n=89) were collected from different centers (Labor Limbach, Heidelberg, Germany; University of Calgary, Canada; Pontifical Catholic University School of Medicine, Porto Allegre, Brazil). All samples were collected according to local ethics committee regulations and stored at −20°C until use.

Synthesis and purification of the C22 peptide

The 22 C-terminal amino acids of the human P2 protein (accession number M17887) were used as template to synthesize the C22 peptide according to the Fmoc-chemistry at the Scientific and Medical Institute Tübingen (NMI, Angewandte F&E, Tübingen, Germany). Quality and purity of the peptide was assessed by mass spectrometry and analytical high performance liquid chromatography (HPLC). The molecular mass was found to be 2,577.92 Da (monoisotopic mass=2,578.05) and had a purity of >98%.

Diagnostic tests

Anti-Rib-P reactivity was characterized using Rib-TriPlex ELISA (Phadia, Freiburg, Germany) composed of a mixture of all three recombinant proteins P0, P1, and P2 [21]. Reactivity to the individual ribosomal P antigens recombinant P0, P1, and P2 and to the synthetic peptide C22 was determined by ELISA as recently described [22].

HCV infection was determined by anti-HCV ELISA (Ortho, Dade-Behring, Deerfield, IL, USA), RecomBlot HCV IgG 2.0 (Mikrogen, Neuried, Germany), and confirmed by polymerase chain reaction (PCR) method. CMV and EBV infection was determined by Enzynost EIA (Dade-Behring, Deerfield, IL, USA) and by EBV/EBNA1 IgG (ESR1362G), VCA IgG (ESR1361G), and VCA IgM ELISA (ESR1361M, Serion Immundiagnostica, Würzburg, Germany), respectively.

Anti-Rib-P positive HCV sera were measured in the semiquantitative ReCombi ANA profile (Phadia, Freiburg, Germany; cut-off ratio 1), containing the autoantigens double stranded DNA (dsDNA), U1 ribonucleoprotein (U1-RNP), Sm, Ro, La, Scl-70, CENP-B, and Jo-1. All assays were performed according to the manufacturer's instructions.

Epitope analysis using immobilized peptides

Solid phase peptides were synthesized using robotics (ASP222; Abimed, Germany). This included a mutational analysis of the C-terminal epitope in which every single residue was successively replaced by alanine (alanine walk). Moreover, we synthesized an epitope scan of consecutive 15mer peptides offset by five amino acids comprising the full length Rib-P2 sequence (accession number M17887) and diverse Rib-P patient-specific epitope-derived peptides [7]. In addition, an amino acid stretch (residues 135–159) of L-APCMH of *Bartonella henselae* (accession number AF484425) was scanned. Furthermore, we compared the reactivity of human and *Trypanosoma cruzi* 13 C-terminal residues of the orthologue ribosomal P proteins. The immobilized peptides were probed with the human sera using an immunoassay as previously reported [7].

NMR analysis of C22

The C22 peptide was dissolved in PBS, pH 6.5 containing 40% trifluoroethanol to a final concentration of 1 mM. NMR experiments were performed on a Bruker DRX600 spectrometer at the European Molecular Biology Laboratory (EMBL, Heidelberg, Germany). 2-D homonuclear nuclear overhauser enhancement spectroscopy (NOESY) with 200 ms mixing time and total correlation spectroscopy (TOCSY) experiments were recorded with 128×2,048 complex points. For solvent suppression, the WATERGATE method was applied [23]. Assignment of the peptide was carried out based on the TOCSY and NOESY experiments, and short proton–proton distances were identified based on the NOESY spectrum. This data was used to shape an approximated molecular model of the C-terminal six amino acids using a molecular visualization and analysis program (GRASP) [24].

Statistical methods

Statistical significance of anti-Rib-P reactivity detected in different patient groups was calculated according to Fisher's exact test. Differences between portions of $p \leq 0.05$ were considered as significant.

Results

Reactivity profile of patients with antiribosomal antibodies

We detected anti-Rib-P reactivity in 34/200 (17%) of SLE patients, in 2/68 (2.9%) patients with HCV infection, and in 0/229 healthy and disease control sera. Fisher's exact test yielded statistical significance for Rib-P reactivity of HCV vs control sera ($p=0.05$) and high significance for SLE vs HCV ($p=0.0013$) and SLE vs control sera ($p=1.05\times10^{-12}$). Of 36 anti-Rib-P positive SLE sera, 17/36 were positive for all 4 Rib-P antigens (P0, P1, P2 and C22), 6/36 for 3, 2/36 for 2, and 6/36 for 1 of the antigens. Two samples from SLE patients reacted exclusively with the C22 peptide, and one serum reacted solely with P0 and P1. 5/36 samples

Table 1 Demographic and serological data of two HCV/Rib-P positive samples

	HCV 5	HCV 43
Demographic data		
Gender	M	M
Age (years)	51	48
HCV (recomBlot HCV 2.0)		
NS3	2+	+
Helicase	2+	+
NS5-12	2+	2+
NS-4	2+	+
Core	2+	2+
Ribosomal P[a]		
C22	0.2	1
P0	0.2	0.8
P1	0.2	1.2
P2	2.4	2.2
No. of positive P2 peptides	3	6
ReCombi ANA profile[a]		
dsDNA	0.2	0.5
U1-RNP	1.8	0.2
Sm	0.2	0.3
Ro52/60	0.4	0.3
La	0.1	0.1
Topoisomerase I	0.2	0.2
CENP-B	0.3	0.4
Jo-1	0.1	0.3

[a] Results in ratio (positive >1.5, borderline 1.0–1.5).

demonstrated the most pronounced reactivity to C22, 10/36 samples to P0, 12/36 samples to P1, and 9/36 samples to P2. The two Rib-P positive HCV samples (HCV-5 and HCV-43) showed pronounced reactivity to ribosomal P2. HCV-43 also reacted with the C22 peptide and with U1-RNP in ELISA (Table 1). None of the anti-Rib-P SLE sera (n=36) was found to be positive for HCV infection (data not shown).

Epitope analysis of antiribosomal P antibodies

The mutational peptide arrays of the C-terminal epitope (alanine walk) was assayed using preselected C22 reactive sera SLE (n=28). The strongest diminishing effect on antibody binding was observed when phenylalanine residues *Phe*^{111} (23/28 sera=82%) or *Phe*^{114} (13/28 sera=46%) were replaced by alanine. All of the sera sensitive to *Phe*^{114} replacement were also sensitive to the *Phe*^{111} mutation (Fig. 1a). Epitope analysis of serum sample HCV-5 and HCV-43 by the SPOT method showed two immunoreactive regions at residues 21–35 but 41–55 of ribosomal P2. Of interest, 4/8 SLE sera reacted with epitope 21–35 and 0/8 SLE sera exhibited reactivity with epitope 41–55. HCV-43 exhibited moderate reactivity to additional peptides that included the major C-terminal Rib-P epitope (Fig. 2). It is interesting to note that one serum from a SLE patient (L41) reacted with an acidic peptide of the Rib-P1 sequence (AAAPAEEKKVEAKKE) and to the C-terminal *T. cruzi* peptide EEEDDDMGFGLFD, but not with the human C22 region EESDDDMGFGLFD. None of the anti-Rib-P sera

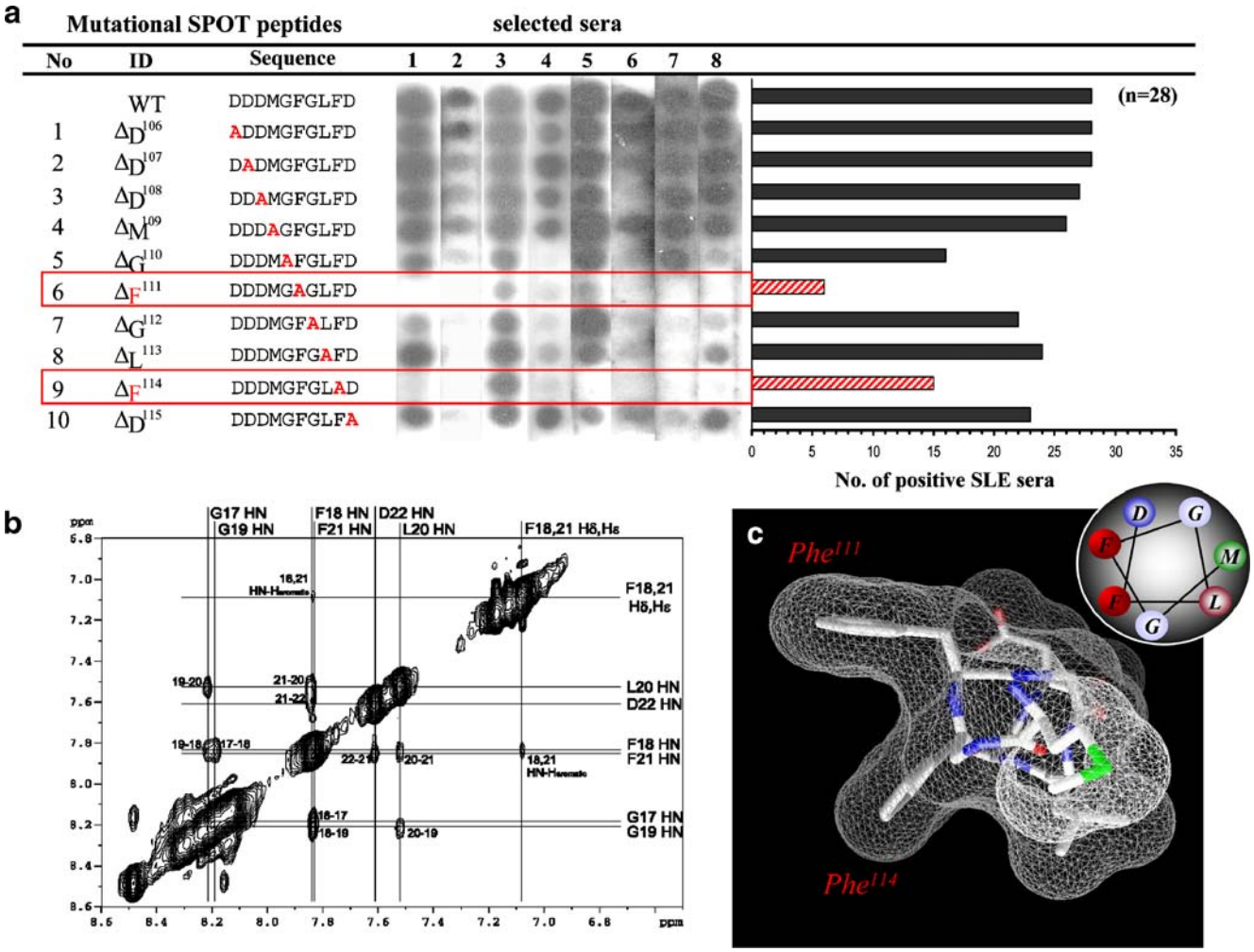

Fig. 1 Mutational and structural analysis of the Rib-P C-terminal epitope based on SPOT peptide and NMR analysis. Mutational analysis of the Rib-P epitope (alanine walk) using anti-Rib-P SLE sera (n=28) confirmed the key role of phenylalanine residues for epitope recognition. Representative SPOT reactivity patterns of selected SLE sera (*1–8*) are shown. *Horizontal bars* represent the number of all sera reacting with the respective peptide (**a**). NMR analysis of C22 peptide reveals hallmarks of an α-helical conformation for the C-terminal epitope core (GFGLFD). The assignment of the chemical shifts is indicated (**b**). Based on the NMR data and on SPOT mutational analyses, an approximated α-helical model of the C-terminal Rib-P epitope core was generated using GRASP. The residues are indicated in the helical wheel scheme (**c**)

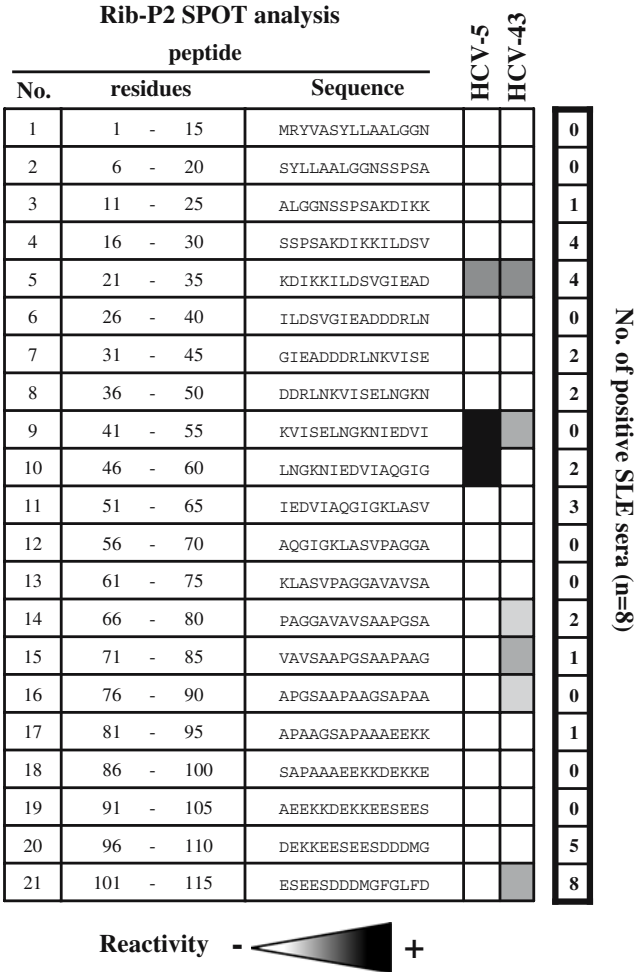

Fig. 2 Epitope mapping of anti-Rib-P2 autoantibodies in the HCV sera. HCV-5 and HCV-43 share reactivity with two epitopes at residues 21–35, which were targeted in 4/8 SLE sera, and 41–55 also targeted by 0/8 SLE sera. HCV-43 also bound the C terminus, which is the major target of the anti-Rib-P SLE sera

demonstrated reactivity to the *Bartonella henselae*-derived peptides containing the defined C-terminal epitope core (GFGLFD) (Table 2).

NMR analysis of C22 peptide

Sequential assignment was readily obtained for the C-terminal six residues (GFGLFD) of the C22 peptide. Inspection of the 2-D NOESY spectrum shows short interproton distances typical for α-helical conformations of the protein backbone, e.g., $H_N(i)$–$H_N(i-1)$ and $H_N(i)$–$H_\alpha(i-1)$ [25]. Furthermore, the H_α chemical shifts of the C-terminal six residues are indicative for an α-helical conformation of the protein backbone, i.e., lower than the random coil chemical shift (Fig. 1b). Based on this, we visualized this stretch including the C-terminal six amino acids as a standard α-helix using GRASP (Fig. 1c) [24].

Discussion

For almost 30 years, anti-Rib-P autoantibodies have been regarded as a specific marker for SLE and they are frequently accompanied by anti-dsDNA and anti-cardiolipin antibodies [6]. Several studies investigated the clinical and serological associations of anti-Rib-P antibodies with the lack of a clear consensus [1, 3–6, 8]. Recent studies strongly supported a direct pathogenic effect of these autoantibodies; observations, which demand the complete elucidation of the humoral autoimmune response to the Rib-P antigen [9, 10]. In the present study, we provide important new insights into the autoantibody response toward the Rib-P antigen.

Using the C22-ELISA, we selected a panel of anti-Rib-P positive SLE sera mainly reacting with the C-terminal epitope of the ribosomal P proteins. This serum panel was then tested on a mutational SPOT peptide array of the C-terminal Rib-P epitope (alanine walk). Most of the sera (23/28) completely lost reactivity when Phe^{111} was replaced by alanine. The second key residue for antibody binding was Phe^{114} (13/28) (Fig. 1a). This observation confirmed the results of a previous study and clarifies the key role of these two residues for autoantibody binding to the C-terminal epitope of the Rib-P antigen [7]. Recently, it has been shown that the key amino acids of the major PM/Scl-100 epitope have a distance of three amino acids [26]. Based on this observation and on structure prediction, a local α-helical secondary structure for the PM/Scl-100 major epitope has been proposed [26]. The distance of three amino acids, as it is present between Phe^{111} and Phe^{114}, is consistent with one turn of an α-helix. Thus, we further investigated the C22 peptide by NMR. The short interproton distances and the H_α chemical shifts of the C-terminal six residues are in fact indicative of an α-helical conformation of the protein

Table 2 Reactivity profile of anti-Rib-P SLE sera with pathogen-derived peptides

Protein	Species	Sequence	No. of positive SLE sera (n=15)
L-APCMH (135–159)	*Bartonella henselae*	QIDGSRGVT*GFGLFD*	0
		GSRGVT*GFGLFD*DRI	0
		GVT*GFGLFD*DRILIW	0
		*GFGLFD*DRILIWPSR	0
Rib-P2	*Trypanosoma cruzi*	EEEDDDM*GFGLFD*	4

Epitope core is indicated using italics

backbone (Fig. 1b). Taken together with the results from the mutational epitope analysis, we propose an α-helical model of the Rib-P C-terminal epitope core. This structural model shows the identified key residues for antibody binding Phe^{111} and Phe^{114} on one side of the helix, probably the side, which is targeted by most of the anti-Rib-P antibodies (Fig. 1c). We additionally provide a theoretical structural model of a single chain variable fragment (scFv) antibody (A4) directed against the Rib-P C terminus, which perfectly reflects the immunological properties known for anti-Rib-P autoantibodies in the context of SLE [27]. The binding site of the scFv A4 displays a characteristic pocket, which could optimally accommodate the C-terminal epitope structure (Fig. S1 in the Electronic supplementary material).

It is certainly of interest that some antibodies from patients with CD also react with the C terminus of the ribosomal P proteins [13]. The recent NMR investigation of peptides derived from the C-terminal 13 residues of the human, *Trypanosoma cruzi*, and *Leishmania braziliensis* ribosomal P proteins showed NOEs congruent with our results [28]. Soares et al. demonstrated that anti-Rib-P antibodies from CD patients require a higher flexibility of the C terminus and show diminished binding to peptides comprising the human sequence of the C-terminal 13 amino acids [28]. These observations suggest clear demarcation of the difference between anti-Rib-P antibodies from patients with CD and those from patients with SLE. Of high interest, we found a serum (L41) sample from a female SLE patient (age 69 years) suffering from arthritis, pleural effusion, cerebral vascular accident with right hemiplegia, bacterial endocarditis, anti-dsDNA, and anticardiolipin (IgG, IgM, IgA) antibodies that reacted with a single peptide of the Rib-P1 sequence AAAPAEEKKVEAKKE and with the C-terminal *Trypanosoma* peptide EEEDDDMGFGLFD, but not with the human C22 region (Table 2). The patient originated from a region in Brazil where CD is epidemic and a previous contact with *T. cruzi* is likely. However, serological evidence for CD is not available because the patient recovered well on metrotrexate and was no longer under clinical observation. Further to the comparison of CD, SLE, and HCV, it is not clear from the other reports if patients with CD had concurrent HCV infection.

A previous homology search using the C-terminal epitope core (GFGLFD) revealed a 100% identical sequence in the L-*iso*-aspasrtyl protein carboxyl methyltransferase homologue of *Bartonella henselae* (accession number AF484425) [7]. This observation was of particular significance because bartonellosis/CSD can lead to SLE-like symptoms [19]. However, none of the SLE sera recognized SPOT peptides comprising the stretch of the *Bartonella henselae* protein containing the epitope core. We assume that here the residues directly N-terminal to GFGLFD counteract the formation of an antigenic motif of the epitope core.

Recent studies have reported a relationship between autoimmune disorders like SLE and HCV infection [15–19]. In a comprehensive study of 134 Spanish SLE patients, Ramos-Casals and colleagues showed that HCV infection can mimic SLE [19]. Most strikingly, we found two samples from male HCV-infected patients with a pronounced reactivity to ribosomal P2 and only moderate (HCV 43) or no binding (HCV 5) to the Rib-P C terminus. Using the ELISA technique, we found that HCV-5 also demonstrated reactivity to another autoantigen (U1-RNP). This observation is in good agreement with the reported autoreactivity of HCV-infected serum samples [29]. To the best of our knowledge, this is the first report of anti-Rib-P autoantibody development in HCV patients without clinical evidence of SLE. HCV is believed to trigger autoantibody development in general via the activation of CD81 on B cells [30]. Because HCV uses the host cell ribosome machinery to replicate, some of the host proteins, ribosomal proteins in particular, may be introduced during the particle assembly. We hypothesize that host self-proteins incidentally introduced in the virus particle may, therefore, be presented to the immune system leading to autoreactivity. Using solid phase peptides, we identified linear epitopes within residues 21–35 and 41–55 of Rib-P2 shared by both HCV sera with anti-Rib-P2 reactivity (Fig. 2). These sequences were aligned with the HCV genome polyprotein (accession number P26664), which contains the amino acid sequences of all antigens tested in RecomBlot HCV 2.0. No significant homologies were found, which contradicts the existence of a linear cross-reactive HCV/Rib-P2 epitope.

Certain SLE-associated autoantibodies including anti-U1-RNPs have been shown to be present before the clinical onset of the disease and thus have high prognostic value [31]. It would be interesting to follow-up anti-Rib-P positive HCV patients to evaluate the prognostic significance of Rib-P2 reactivity for the future onset of SLE. A heightened prevalence of HCV infection has indeed been described in SLE patients [15]. However, no HCV positive sera were found in our panel of anti-Rib-P positive SLE sera (n=28). The cohort was too small to allow any statistically relevant conclusion, but we assume no further increased correlation of HCV infection with the occurrence of anti-Rib-P autoantibodies in SLE sera.

Based on our results, we provide a structural model of the C-terminal Rib-P epitope and are the first to report anti-Rib-P autoantibody development in the context of HCV infection. We further conclude that the humoral autoimmunity to the ribosomal P proteins is diverse in autoimmunity and infection. Although the majority of anti-Rib-P positive samples bind to the C-terminal epitope and are sensitive to phenylalanine replacements in this region, other linear and conformational epitopes may play an important role for the self-reaction to this protein complex. Of note, 2/68 HCV positive sera with

anti-Rib-P2 share reactivity to epitope regions located within the N-terminal half of the P2 protein. Further studies including longitudinal analyses are required to clarify a putative role of HCV in the initiation of the self-reactivity to the ribosomal P proteins and the development of SLE.

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Competing interests None declared.

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