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Research Article

Recombinant anti-P protein autoantibodies isolated from a human autoimmune library: reactivity, specificity and epitope recognition

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Abstract. The ribosomal P proteins are specific and important autoantigens in patients affected by systemic lupus erythematosus. In this study, we describe for the first time the selection and characterization of recombinant human monoclonal anti-P protein (auto)-antibody fragments from an autoimmune patient-derived phage display antibody library. The selected recombinant anti-P antibodies specifically recognize the P proteins in immunofluorescence assays on HEp-2 cells and in immunoblotting assays, and they immunoprecipitate the P proteins under native conditions. Using both anti-P-positive patient sera and the selected recombinant anti-P antibodies, the immunodominant epitope was determined and shown to be located at the C-terminal end of the P proteins (amino acids 111–115). Inhibition of in vitro protein translation demonstrated that interaction of the monoclonal patient-derived anti-P antibodies with their native epitope functionally inhibits the activity of the P proteins on the ribosome, confirming the notion that patient autoantibodies are often directed to the functional centre of their autoantigenic target.

Key words. Recombinant antibody fragment; P protein; scFv; epitope mapping; autoantibody.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of high titres of anti-nuclear and anti-cytoplasmic antibodies in patient sera. These antibodies and their respective antigens form immune complexes that can deposit in the tissue generating injuries characteristic of the disease.

A large number of autoantibodies are associated with SLE including those directed to the ribosomal phospho (P) proteins [1]. The anti-P antibodies target the three P proteins named P0, P1 and P2, which are located as a pentameric complex on the larger subunit of the eukaryotic ribosome. The complex is formed by two dimers of P1 and P2 and one monomer of P0 that mediates the binding of the complex to the ribosomal stalk [1, 2]. P1 and P2 proteins interact through their N-terminal domain with P0 [3–5].

The ribosomal P complex forms a GTPase domain to which the eukaryotic elongation factor eEF-2 binds during protein synthesis [1, 6]. The GTPase activity of eEF-2, which catalyses the translocation of the peptidyl-tRNA from the A to the P site of the ribosome, is dependent on the presence of P1 and P2 on the large ribosomal subunit

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[7]. The interaction of both P1 and P2 with eEF-2 results in a conformational change of the factor that triggers its GTPase activity [8].

Approximately 15–20% of Caucasian and up to 40% of Asian patients with SLE develop autoantibodies against the P proteins [9–11]. The observed frequency of the anti-P antibodies in patient sera further depends on the assay used to detect the antibodies – the most sensitive method is the immunoblotting technique [12–15]. The titre of these antibodies appears to correlate with disease activity, and clinical associations with lupus hepatitis or glomerulonephritis and psychosis have been described [reviewed in ref. 16].

The anti-P antibodies recognize both conformational and linear epitopes [17]. The immunodominant epitope is contained within the C-terminal 22-amino-acid-long (C-22) sequence [1], protruding from the ribosomal stalk, common to the three P proteins [18]. The carboxy-terminal end is highly conserved in eukaryotic P0, P1 and P2. The C-22 peptide is the main common requirement for basic stalk activity, probably due to its interaction with some conserved region in the elongation factor [19].

The P1 and P2 proteins are phosphorylated [1] on serine residues when present on the ribosome and this phosphorylation seems to be important for their function and to mediate the binding of the complex to the ribosome [20–23]. During Fas-L-induced apoptosis, the P proteins are dephosphorylated [24] and this modification could induce 'neo' epitopes that might trigger the autoimmune response in SLE.

Due to the clinical relevance of the anti-P antibodies, more knowledge about their epitope recognition and fine specificity is required. For this purpose, recombinant human anti-P single-chain (auto)-antibody fragments (scFvs) were isolated from an autoimmune patient-derived library using phage display technology [25, 26]. The selected monoclonal anti-P antibodies were compared with a number of patient anti-P-reactive sera for their reactivity, fine specificity and epitope recognition. One recombinant antibody was also functionally tested for its ability to inhibit the activity of the P proteins on the ribosome.

Materials and methods

Patient serum samples

Sera from ten adult SLE patients (D18, K33, L8, M26, M88, 11167, 23646, 0217, 10026 and 10955) which fulfilled the American College of Rheumatology 1982 revised criteria for the classification of SLE [27] were used in this study.

D18, K33, L8, M26 and M88 patients were followed at the Department of Rheumatology and Internal Medicine of the University Medical Centre St. Radboud (Nijmegen, The Netherlands). Patient samples 11167, 23646 and 0217 were taken from the serum bank at Pharmacia Diagnostics (Freiburg, Germany). Patients 10026 and 10955 were followed at the Division of Rheumatology, Department of Medical and Surgical Science of the Central Hospital, Padova (Italy). All human sera contained polyclonal antibodies directed towards the C-terminal end of the P proteins.

Selection and purification of the antibody fragments

The human library was derived from bone marrow and peripheral blood lymphocytes of five SLE patients (D18, D5, O11, Z5 and S339) and constructed in the pHENIXVSV vector [28] as described by Hoet et al. [29, 30]. This vector contains a vesicular stomatitis virus (VSV-G) tag that is recognized by the mouse anti-VSV-G-tag monoclonal antibody P5D4 [31]. Separate libraries were constructed from each individual patient. The phage repertoire was panned in two successive selections for binders as described previously [32], using 10 µg of the C-22 peptide conjugated to bovine serum albumin (1:2 w:w using 0.2% glutaraldehyde. Library D18 (complexity ~1.0×10^8) was used separately in the first selection, and a pool of all libraries (complexity of individual libraries: D5 ~1.9×10^8, O11 ~0.38×10^8, Z5 ~1.6×10^8, S339 ~0.01×10^8) was used in the second selection. Phages were screened for binding to the C-22 peptide in an ELISA assay as described elsewhere [33]. To test the specificity of the selected anti-P clones, maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with a set of recombinant autoantigens (U1A, 34 kDa; annexine XI, 56 kDa; Ro/SSA 60 and 52 kDa; La/SSB 50 kDa; Jo-1, 54 kDa; DNA topoisomerase I, 100 kDa) in 50 mM $NaHCO_3$, pH 9.6 (100 µl/well, 10 µg antigen/ml). C-22-positive clones were sequenced and the obtained sequences were compared with the human germline sequences in the V-BASE sequence database using the software at http://www2.ebi.ac.uk/clustalw/ (EMBL-European Bioinformatics Institute, Wellcome Trust Genome Campus, Cambridge, UK).

Individual C-22-positive clones were cloned into the expression vector pUCmκ-VH6 containing the mouse Ig light-chain Cκ domain [Raats et al., unpublished results], and the Fos-dimerization-domain-containing vector pUFosVH6 [28].

Indirect immunofluorescence staining

HEp-2 cells grown on slides were fixed in methanol (15 min at –20 °C) and acetone (5 min at –20 °C) and air-dried. Cells were incubated for 1 h at room temperature (RT) with scFv anti-P antibody, human anti-P-positive serum and the appropriate controls diluted 1:50 and 1:20, respectively, with PBS. The slides were then washed three times with PBS and bound recombinant antibodies were detected with anti-VSV-G-tag monoclonal

antibody in PBS, for 1 h at RT followed, after three washes with PBS, by incubation for 1 h at RT with FITC-conjugated anti-mouse or anti-human IgG antibodies (Dako, High Wycombe, UK) diluted 1 : 50 with PBS. The slides were mounted in 50% glycerol and examined under a fluorescent microscope.

Analysis of cell extracts

Jurkat (human T cell leukaemia) suspension cells were grown and total cell extract obtained as previously described [34]**.** Total cell extracts from 1×10^6 Jurkat cells and P proteins immunoprecipitated from these extracts by scFv anti-P antibodies were size-fractionated by 15% SDS-PAGE and blotted onto nitrocellulose filters (Schleicher & Schuell). Immunodetection of the P proteins was performed using the recombinant anti-P antibodies and the 10026 serum as previously described [34].

Epitope mapping

To characterize the epitope recognition of the selected recombinant antibodies, 16 synthetic peptides were generated shortening the C-22 linear peptide by one amino acid starting from the N- or C-terminal end (table 1). The C-22 amino acid sequence was deduced from the cDNA sequence of human P2 protein [6]. Peptides were synthesized by the solid-phase strategy as described previously [35].

The shortened synthetic peptides were coupled covalently overnight (o/n) to 96-well assay plates (Costar Corporation, Cambridge, Mass.) at 10 µg/well in PBS pH 9.0, 0.5 M NaCl, essentially as described elsewhere [35]. The plates were then incubated for 1 h at RT with 100 µl recombinant antibody 1 : 1 diluted in 5% non-fat dried milk powder in PBS (MPBS) or 10026 human serum 1 : 200 diluted in 1% BSA/RIA buffer (0.5 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS). After 3 × 5 min washes with PBS-0.05% Tween 20 (PBS-T) and 3 × 5 min with PBS, anti-VSV-G-tag monoclonal antibody in 5% MPBS was added for 1 h at RT. After extensive washing, bound recombinant antibodies were detected using HRP-conjugated rabbit anti-mouse IgG antibodies (Dako, High Wycombe, UK) diluted 1 : 200 in 5% MPBS, and bound human anti-P antibodies with anti-human IgG HRP-conjugated antibodies (Dako) diluted 1 : 1000 in 1% BSA/RIA for 1 h at RT. The plate was washed again and the antibody binding was visualized using K-Blue substrate (NEOGEN, Lexington, Mass.).

To characterize the antibody specificity in more detail we used the SPOT method to perform a mutational analysis of the major ribosomal P epitope recognized. For the spot blots, membrane-bound peptide libraries of the C-terminal 10-amino-acid-long (C-10) peptide (DDDMGFGLFD) were synthesized with a pipetting ro-

Table 1. Amino acid sequences of the 16 synthetic peptides used for the epitope mapping.

Peptide	Sequence	Peptide recognition		
		A4	C10	positive serum
C-22	NH_{2-94} EKKDEKEESEESDDDMGFGLFD$_{115}$-COOH	+++	+++	+++
27	ESEESDDDMGFGLFD	+++	+++	+++
26	EESEESDDDMGFGLF	+	+	++
25	KEESEESDDDMGFGL	+	+	++
24	KKEESEESDDDMGFG	+	+	++
23	EKKEESEESDDDMGF	+	+	+
22	EEKKEESEESDDDMG	–	–	–
21	KEEKKEESEESDDDM	–	–	–
20	KKEEKKEESEESDDD	–	–	–
26b	SEESDDDMGFGLFD	+++	+++	+++
27b	EESDDDMGFGLFD	++	++	++
28	ESDDDMGFGLFD	++	++	++
31	SDDDMGFGLFD	+	++	++
34	DDDMGFGLFD	+	+	++
48	DDMGFGLFD	+	+	+
51	DMGFGLFD	+	+	+
52	MGFGLFD	+	+	+

All 16 synthetic peptides were covalently coated on ELISA plates and the recombinant autoantibodies and human anti-P-positive serum were screened for their recognition of the different peptides.

bot (ASP222 machine; Abimed, Langenfeld, Germany) according to the protocol described by Gausepohl and Behn [36]. The spot blot immunoassay was performed as described previously with minor modifications [37]. Briefly, following completion of peptide synthesis, non-specific binding sites were blocked by o/n incubation of the membranes in blocking buffer (2% Marvel in PBS). After one washing step, the membranes were incubated with recombinant antibody fragments (present in bacterial culture supernatant) or patient serum diluted 1:4 and 1:200, respectively, in blocking buffer for 2 h at RT. Unbound antibodies were removed by three washing steps in PBS-0.2% Tween-20. Bound recombinant antibodies were detected using HRP-conjugated rabbit anti-mouse IgG antibodies (Dako) diluted 1:200 in 5% MPBS, and bound human anti-P antibodies with anti-human IgG HRP-conjugated antibodies (Dako) diluted 1:1000 in 1% BSA/RIA for 1 h at RT. Antibody binding was visualized by chemiluminescense as previously described [34].

In vitro translation

To test whether recombinant antibodies reacted with the functional domain of the P proteins, their ability to inhibit protein synthesis was measured. In vitro translation was performed in a cell-free system using rabbit reticulocyte lysate (TnT Coupled Reticulocyte Lysate Systems; Promega Madison, Wis.) in the presence of ^{35}S-methionine, as described by the manufacturer. The DNA control from the kit was used for transcription/translation of luciferase, a 61-kDa protein. The A4 recombinant anti-P antibody and a non-ribosome-related control recombinant antibody Ra3 [Raats et al., unpublished results] were added to the translation reaction at six different concentrations. Proteins were then fractionated on 10% SDS-PAGE and the gels were subsequently treated with Amplify (Amersham, UK) and analysed by fluorography.

Results

Selection of human anti-P antibody fragments

SLE-autoimmune-patient-derived antibody phage display libraries [29, 30] were screened for the presence of human antibody fragments specific for the ribosomal P proteins. Anti-P antibodies were obtained in the successive selections after two and three rounds of panning, using the C-22 BSA-conjugated peptide as antigen. Ninety-six colonies from the third and 48 colonies from the second and the fourth rounds were tested in ELISA for P protein binding. Although many positive clones were found, based on the *Bst*NI restriction enzyme pattern, only two different anti-P-reactive clones (one from each selection) could be identified, referred to as clones A4 and C10.

Specificity and germline family

The specificity of the selected antibody fragments was determined by ELISA using a control panel of recombinant autoantigens (U1A, annexin XI, Ro/SS-A, La/SS-B, Jo-1, DNA topoisomerase I). Both antibodies reacted only with the C-22 peptide and not with the control autoantigens (data not shown).

The selected cDNA clones were sequenced and the DNA sequence compared with germline sequences, as depicted in figure 1. Both recombinant anti-P antibodies appeared to be derived from the VH3 heavy-chain germline family. The A4 clone could be aligned with the VH3 locus 3-30.3 (gene DP-46) and the C10 clone with the VH3 locus 3-23 (gene DP-47). Except for the CDR3 region, the two clones are similar and the DNA sequences are also very close to their cognate germline gene sequence. Some mutations were observed: the mutations on the A4 clone sequence were distributed throughout the CDR1/2 and FR1/2/3 [13 mutations in total (1 PCR oligo introduced)]. Remarkably, only one somatic and three PCR oligo-introduced mutations were found in the C10 clone VH sequence (fig. 1).

The light-chain germline sequences of the A4 and C10 clones were derived from the Vk3 L16 and VL3 3l locus, respectively. The observed somatic mutations (15 in the A4 and 9 in the C10 gene) were distributed throughout CDR1,2,3 and FR3 for the A4 sequence, and throughout the CDR1/3 and FR3 regions in the C10 sequence (fig. 1).

To increase the avidity (by converting the single chains to dimers) and to facilitate detection of the selected scFv fragments, they were cloned into pUFosVH6 and pUCmκ-VH6 vectors.

Indirect immunofluorescence staining, Western blotting analysis and immunoprecipitation

The indirect immunoflescence (IIF) staining on HEp-2 cells obtained with recombinant anti-P-positive clones and the patient serum as control is shown in figure 2. The recombinant antibodies revealed an intense cytoplasmic staining (fig. 2a, b) identical to the pattern obtained with the patient serum (fig. 2c).

The recombinant anti-P antibodies, non-ribosome-related anti-La/SSB antibody fragment (5D3) [38] and the human anti-P-positive patient serum were also tested in an immunoblotting assay with Jurkat total cell extract. The recombinant anti-P autoantibodies reacted strongly with the P proteins, as shown in figure 3, lanes 2 and 3. The A4 antibody predominantly stained the P0 and the P1 proteins on the blot (fig. 3, lane 2), while the C10 antibody was able to recognize all three P proteins (fig. 2, lane 3). This difference between the two recombinant antibodies appeared to be independent of the dilution of the scFvs, suggesting small differences in epitope recognition. The anti-La/SSB recombinant antibody

Heavy chain V-genes

Clone	Family/Locus	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	mutations*
		1 2 3		4	5 6	7 8 9			
		12345678901234567890123456789 0	1ab2345	678901234567 89	012abc3456789012345	67890123456789012abc345678901234			
	VH3/3-30.3	qvqlvesGGGVVQPGRSLRLSCAASGFTFS	S--YAMH	WVRQAPGKGLEWVA	VISY--DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR			
A4		.....q.....K.....S..	R--KS..	.....L.	....--..TKE.....	.....H.....V.....T	SSEGVHGFDV	WGQgtmvtvstss	12(1)[D4-23/JH3a]
		***** :********* :********** :**	-- :**	********** :*	****--**:::********	********** :******* .*********			
	VH3/3-23	evqllesGGGLVQPGGSLRLSCAASGFTFS	S--YAMS	WVRQAPGKGLEWVS	AISG--SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK			
C10		q...vq.....	.--....	.....	....--.....	.....R	APVKNYYDSRGYYRSGAFGI	WGQgtmvtvstss	1(3)[D3-22/D21-9/JH3b]
		:***::**********	*--****	**********	****--**********	********** :*******			

Light chain V-genes

Clone	Family/Locus	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	mutations*
		1 2	3	4	5	6 7 8	9		
		12345678901234567890123	45678901abcdef234	567890123456789	0123456	78901234567890123456789012345678	9012345		
	VK3 L16	eivmtqspATLSVSPGERATLSC	RASQSVSS------NLA	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYNNWP		
A4		...l.....	.....R.------..V	.....	D..N..S	.V.P.....D.....	...TQ..PSIT	FGggtkveikr	14(1) [JK4]
		:***************	****** *------**.	**********	.**.**:	*:*.*********:******************	***.:**		
		12345678912345678901 23	45678901abc234	567890123456789	01abcde23456	7890123456 78ab9012345678901234 5678	9012345abcde		
	VL3 3l	sseltqdPAVSVALGQTVRITC	QG-DS-LRSY-YAS	WYQQKPGQAPVLVIY	GK-----NNRPS	GIPDRFSGSSSG--NTASLTITGAQAEDEADYYC	NSRDSSGNH		
C10		l.....	..-..-..T.-..N	.....	..-----.....	.....I..--.....G.....	.F.....YLV	FGggtqltvls	8(1) [JL2/JL3a]
		**********	**-**-**:*-**.	**********	**-----*****	********* **--********** *******	* ******:		

Figure 1. Amino acid sequences of the heavy and light chains of anti-P antibody scFvs aligned to their homologous germline sequence. Sequence alignment of the anti-P recombinant antibodies with the most homologous germline genes (Clustal W alignment). Dots indicate amino acids that are identical to those of the respective germline gene; dashes indicate gaps. Symbols in the Clustal W consensus lines: * marks identical or conserved amino acids in all sequences in the alignment; indicates conserved substitutions; indicates semi-conserved substitutions. Lowercase amino acids correspond to regions introduced by the oligonucleotides used for amplification of the VH and VL fragments. Framework and CDR regions are indicated by FR1, FR2, FR3, FR4, CDR1, CDR2 and CDR3. Mutations*: oligonucleotide-introduced mutations are indicated as (n). D/J recombination is indicated as [D/J].

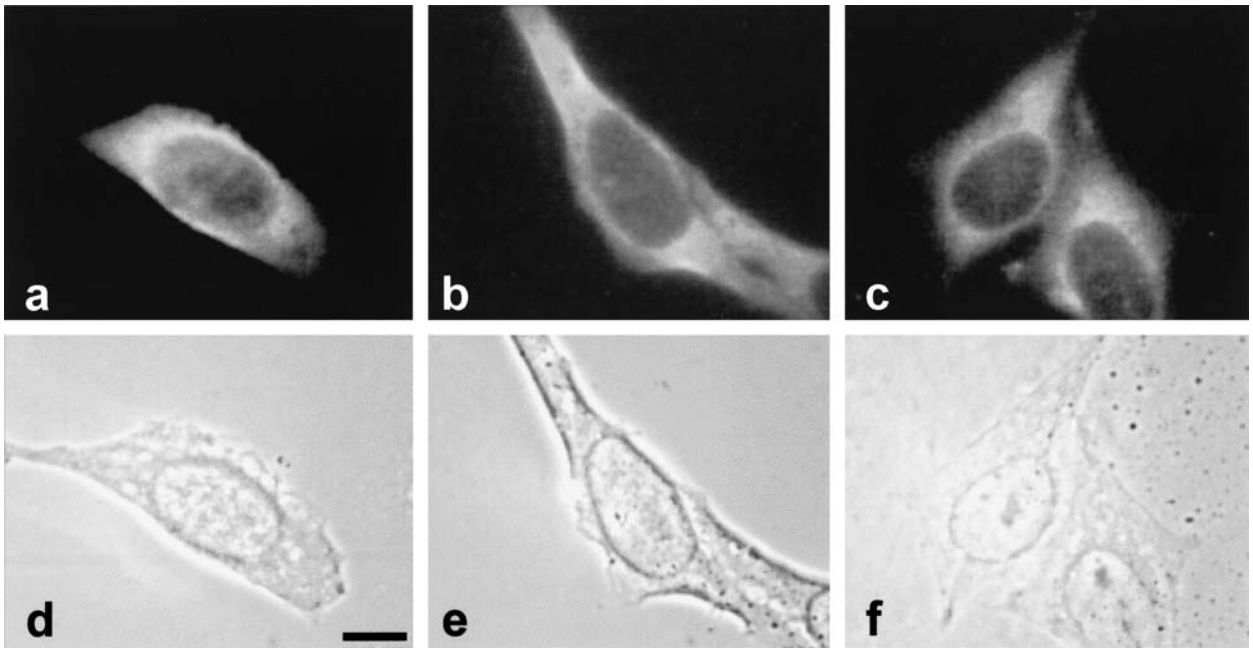

Figure 2. IIF staining with the recombinant anti-P antibodies. The recombinant anti-P antibody A4 (*a*) and C10 (*b*) were used to immunolocalize the P proteins on HEp-2 cells. The 10026 anti-P-positive SLE patient serum was used as control (*c*). The recombinant anti-P antibodies showed the typical cytoplasmic staining (*a, b*), comparable to that given by the patient serum (*c*). (*d–f*) Morphology of the corresponding cells. Bar, 10 µm.

reacted specifically with the La protein on the blot (fig. 3, lane 4).

ScFv antibody fragments bound to protein A-agarose were also tested for their ability to immunoprecipitate P proteins from total Jurkat cell extract [34]. The immunoprecipitated proteins were analysed by immunoblotting using the 10026 anti-P-positive patient serum as probe. Both recombinant antibodies were able to immunoprecipitate the whole P protein complex (fig. 4, lanes 2, 3) whereas the non-ribosome-related clone was not (fig. 4, lane 4). These results show that the P complex antigen is recognized under native conditions by the anti-P selected antibodies.

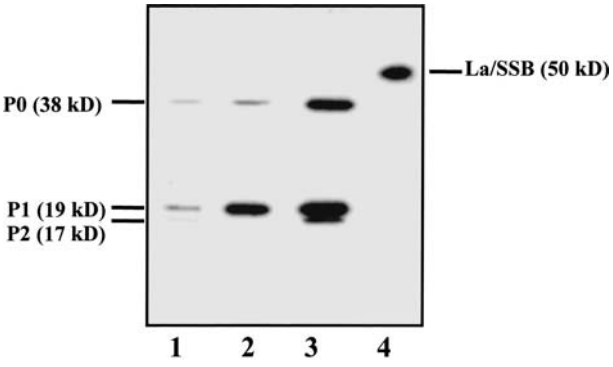

Figure 3. Reactivity on immunoblot of the recombinant anti-P antibodies. Jurkat total cell extract from 1×10^6 cells/lane was size fractionated by 15% SDS-PAGE, blotted onto a nitrocellulose membrane and probed with the 10026 anti-P-positive SLE patient serum (lane 1), the A4 (lane 2), and C10 (lane 3) recombinant anti-P antibodies, and the non-related anti-La/SSB recombinant antibody (lane 4) as negative control. The bound recombinant antibodies were detected using an anti-VSV-G-tag HRP-conjugated secondary antibody diluted 1:5000 in 5% MPBS. The reaction was visualized via chemiluminescence. The A4 recombinant antibody recognized the P0 and the P1 proteins, and C10 recognized all three P proteins. The anti-La/SSB recombinant antibody specifically recognized the La protein as indicated on the right of the figure. The recognition pattern of the scFv antibodies is comparable to that obtained with the patient serum used as a positive control.

Epitope mapping

To compare the epitope recognition of the selected anti-P antibodies with that of antibodies from patient serum, 16 synthetic peptides generated from the C-22 sequence were coated onto ELISA plates (see Materials and methods). The plates were then incubated with the recombinant antibodies or the human serum and the reactivities were compared. Both recombinant anti-P and patient serum antibodies failed to recognize the peptides lacking the last five C-terminal amino acid residues 111–115 (table 1, peptides 22, 21 and 20).

In contrast, all the peptides lacking N-terminal amino acid residues were efficiently recognized by both the scFvs and the patient serum antibodies (table 1, peptides 26b–52). These results indicate that the scFvs, just like the patient antibodies, recognize a linear epitope contain-

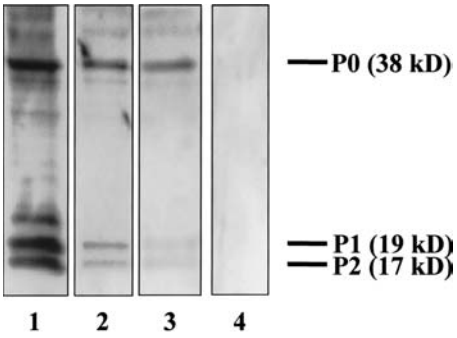

Figure 4. Immunoprecipitation with the recombinant anti-P antibodies. P proteins were immunoprecipitated from Jurkat total cell extract using the A4 (lane 2) and C10 (lane 3) recombinant antibodies and the non-related La/SSB recombinant antibody (lane 4) coated to protein-A beads via anti-mouse and anti-VSV-G-tag antibodies. The immunoprecipitated P proteins were fractionated by 15% SDS-PAGE, blotted onto a nitrocellulose membrane and detected on the blot using the 10026 (all lanes) anti-P-positive SLE patient serum as probe. Lane 1 corresponds to the input and the position of the three P proteins is indicated on the right.

ing the very C terminal end of the P proteins.

To determine the fine specificity of the epitope recognition of the selected recombinant antibodies as well as of anti-P-positive patient sera, we performed a mutational analysis of the major epitope recognized by the anti-P antibodies using peptide arrays prepared by the SPOT method (fig. 5). For scFv A4, the amino acids Phe^{111}, Leu^{113}, Phe^{114} and Asp^{115} could be identified as the key residues for the interaction (fig. 5A). In contrast, scFv C10 showed a more unspecific recognition pattern. Only Phe^{114} and Asp^{115} could not be replaced by other amino acid residues without a loss in reactivity (fig. 5A). The immune response towards the C10 peptide was quite diverse among the patients (fig. 5B). Nevertheless, some similarities could be observed. Four out of seven sera tested mainly contained autoantibodies towards the epitope core Gly^{110}, Phe^{111} and Gly^{112} with a further contribution of Phe^{114} (11167, 23646, L8 and 0217). In contrast, the epitope of a second group was found to be located at the very C terminal part of the C10 peptide, varying in length (M88, M26 and K33).

The A4 scFv antibody inhibits translation

To test whether the recombinant anti-P antibody A4 was able to interact with the functional domain of the P proteins, its ability to block protein synthesis in vitro was measured. Since both antibodies showed very similar epitope reactivity patterns, only clone A4 was analysed. The recombinant antibody fragment blocked the activity of the P proteins very efficiently, resulting in complete inhibition of the translation process (fig. 6A, lane 1). Complete inhibition of luciferase synthesis was obtained when the recombinant anti-P antibody was added to the reaction mixture at a concentration of 920 ng/µl (fig. 6A, lane 1). At the same concentration, the non-ribosome-related control antibody Ra3 showed no effect (fig. 6B, lanes 1–6).

Discussion

The three ribosomal phospho proteins P0, P1 and P2 are targetted by autoantibodies in SLE patients [1]. The anti-P antibodies are considered to be specific markers for this disease and their presence is strongly associated with disease activity and with some clinical manifestations [16]. Anti-P antibodies can bind to the cellular membrane and have actually been shown to penetrate into a variety of cultured cell lines, providing some evidence for a possible pathogenic role of these antibodies. The pathogenic mechanism of the anti-P autoantibodies might be related to the presence of the P proteins on the surface membrane of many cells, especially actively proliferating cells [39]. The anti-P antibodies appear to interact with a membrane form of the P0 protein, and after binding they can be internalized into the cell where they can reach the P complex located on the ribosomes. The activity of the P protein complex is then blocked with the consequence that protein synthesis is severely affected. The penetration of mouse monoclonal anti-P antibodies into living cells can induce apoptosis [40].

In an attempt to study individual human monoclonal anti-P antibodies, we isolated and characterized human recombinant scFvs specific for the P proteins from an SLE autoimmune patient-derived antibody phage display library [25]. Remarkable was the observation that, in two separate selections, many anti-P-positive scFvs could be identified by ELISA, but after fingerprint analyses and sequencing, only two very dominant clones were found, one from each selection. The particular conformation of the epitopes recognized by these antibodies possibly does

Figure 5. Mutational analysis of the major epitope recognized by the anti-P antibodies. The C-10 peptide sequence was used to synthesize peptide libraries according to the SPOT method. Membranes were probed with recombinant anti-P antibodies (*A*) and human anti-ribosomal sera (*B*). Four out of seven anti-P protein patient sera tested contain antibodies directed against the central part of the epitope with Gly^{110}, Phe^{111} and Gly^{112} being the key residues (serum 11167, 23646, L8 and 0217). The other sera are more specific for the most C terminal part of the epitope (M88, M26, K33). Except for one serum (M88), no significant contribution to the antibody binding could be observed by the acidic stretch of three Asp residues. For scFv A4, the amino acids Phe^{111}, Leu^{113}, Phe^{114} and Asp^{115} could be identified as the key residues for recognition. ScFv C10 displayed a more unspecific recognition pattern. Phe^{114} and Asp^{115} could be replaced by only a few amino acid residues and thus represent the key residues for scFv C10.

A
C10
A4
B
11167
L8
23646
M88
M26
0217
K33
reactivity
D D D M G F G L F D
106 107 108 109 110 111 112 113 114 115
A R N D C Q E G H I L K M F P S T W Y V

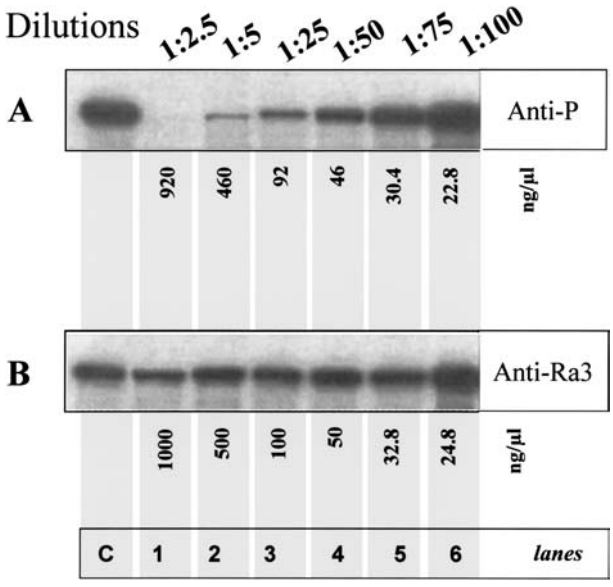

Figure 6. In vitro translation experiments. The A4 recombinant anti-P antibody (*A*) and another non-ribosome-related recombinant antibody (Ra3) (*B*) were added to the translation reaction in the presence of ^{35}S-methionine, at six different dilutions as reported at the top of the figure. The corresponding concentration is indicated at the bottom of the blots. Proteins were size fractionated by 10% SDS-PAGE and the reaction visualized by fluorography. The band on the gels corresponding to the luciferase protein used for the translation was not visible when 920 ng/µl of the recombinant anti-P antibody was added to the translation reaction (lane 1) (*A*). The luciferase band was more evident when the concentration in the translation mixture of the recombinant anti-P antibody was lower (lanes 2–6). No decrease in the signal was observed when the non-ribosome-related recombinant antibody (lanes 1–6) (*B*) was used. In the control lane C, no recombinant antibody was added.

not permit the selection of many different types of scFv clones. Moreover, the two selected scFvs were derived from the same VH3 germline gene family. ScFv A4 has a medium mutation frequency compared to recombinant antibodies selected from other autoimmune-patient-derived phage display libraries [29, 32, 34]. In contrast, the VH sequence of scFv C10 contains a relatively long CDR3 region and is almost identical to the germline sequence. The low number of mutations might indicate the presence of autoreactive naive B cells in these SLE patients. Furthermore, both VL genes contain average numbers of mutations compared to previously isolated recombinant autoantibodies [29, 32, 34].

The selected recombinant anti-P antibodies specifically recognize the P proteins in immunoblotting. Recombinant antibody A4 recognizes P0 and P1, while antibody C10 recognizes all three P proteins. This is in agreement with results with polyclonal patient anti-P antibodies: some patient sera contain anti-P antibodies recognizing all three P proteins on Western blot, whereas some recognize only one or two P proteins. This might be due to the fact that the targeted epitopes, although located within the C-22 terminal end that is common to the three P proteins, also depend on sequences which are more N terminally located [14]. Since we did not observe differences in the recognition of the C-terminal epitope (for both clones, amino acids 111–115 are critical for recognition), we assume that some subtle sequence changes in the more N terminal regions of the P proteins determine their different recognition by the scFvs on immunoblots. Via epitope characterization at the amino acid level using SPOT technology, although the two selected recombinant antibody fragments bind to the same region around amino acids 112–115 (of the human P2 sequence), they display a different fine specificity. Since scFv A4 proved to be more specific for the major ribosomal P epitope, as revealed by a lower number of tolerated amino acid substitutions, one might speculate that this clone represents a more evolved antibody.

The finding that the scFvs immunoprecipitate the P protein complex from a total cell extract and are able to immunolocalize the P proteins in the cytoplasm of HEp-2 cells indicates that these recombinant anti-P antibodies recognize the native epitope. The immunofluorescence pattern of the selected antibodies is virtually identical to that observed for anti-P antibodies contained in the SLE patient serum used as a control (compare fig. 2a and b to c). A weak staining of the nucleoli was only observed using the patient serum and not with the recombinant antibodies, possibly because the former contains, in addition to anti-P antibodies, autoantibodies recognizing nucleolar-localized antigens such as PM/Scl or fibrillarin.

We can conclude from our results that the recombinant anti-P antibodies are very specific monoclonal anti-P protein antibodies that mimic the patients' polyclonal antibody response.

The last ten amino acid residues of the P proteins are known to be very conserved in all eukaryotes, suggesting that the C terminal end has a very important role in the function of these proteins [41]. This part of the P proteins is exposed and interacts with the elongation factor eEF-2 during the synthesis of proteins [1, 6].

We have shown that the recombinant anti-P antibody A4, but not the control scFv, inhibits the in vitro translation of luciferase in a reticulocyte cell-free system, most likely by interacting with the functional domain of the P proteins. This experiment not only confirms that the P proteins are essential for ribosome activity during translation, but also corroborates the notion that patient autoantibodies are often directed to the functional centre of their autoantigenic target. This has been shown previously for the scleroderma-specific autoantibodies directed to DNA topoisomerase I [42] but also for other autoantibodies such as those directed to the U1-70K protein (inhibition of splicing) and for the myositis-specific antibody Jo-1 (inhibition of charging of His-tRNA) [43]. In future stud-

ies, these recombinant anti-P antibodies will be applied for exploring the pathogenic role of the anti-P antibodies in systemic lupus erythematosus**.**

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1 Elkon K. (1994) Ribosomal RNP. In: Manual of Biological Markers of Disease, B 2.5, pp. 1–11, Venrooij van W. J. and Maini R. N. (eds), Kluwer, Dordrecht
2 Uchiumi T., Wahba A. J. and Traut R. R. (1987) Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking. Proc. Natl. Acad. Sci. USA. **84:** 5580–5584
3 Möller W. (1990) The ribosome: structure, function and evolution. Am. Soc. Microbiol. Washington DC 380–389
4 Lijas A. (1991) Comparative biochemistry and biophysics of ribosomal proteins. Int. Rev. Cytol. **124:** 103–135
5 Rodriguez-Gabriel M., Remacha M. and Ballesta J. P. G. (2000) The RNA interacting domain but not the protein interacting domain is highly conserved in ribosomal protein P0. J. Biol. Chem. **275:** 2130–2136
6 Rich B. E. and Steitz J. A. (1987) Human acidic ribosomal phosphoproteins P0, P1 and P2: analysis of cDNA clones, in vitro synthesis, and assembly. Mol. Cell. Biol. **7:** 4065–4074
7 Lavergne J. P., Conquet F., Reboud J. P. and Reboud A. M. (1987) Role of acidic phosphoproteins in the partial reconstitution of the active 60 S ribosomal subunit. FEBS Lett. **216:** 83–88
8 Bargis-Surgey P., Lavergne J. P., Gonzalo P., Vard C., Filhol-Cochet O. and Reboud J. P. (1999) Interaction of elongation factor eEF-2 with ribosomal P proteins. Eur. J. Biochem. **262:** 606–611
9 Elkon K. B., Parnassa A. P. and Foster C. L. (1985) Lupus autoantibodies target ribosomal P proteins. J. Exp. Med. **162:** 459–471
10 Yoshio T., Masuyama J., Ikeda M., Tamai K., Hachiya T., Emori T. et al. (1995) Quantification of antiribosomal P0 protein antibodies by ELISA with recombinant P0 fusion protein and their association with central nervous system disease in systemic lupus erythematosus. J. Rheumatol. **22:**1681–1687
11 Ghirardello A., Doria A., Zampieri S., Gerli R., Rapizzi E. and Gambari P. F. (2000) Anti-ribosomal P protein antibodies detected by immunoblotting in patients with connective tissue diseases: their specificity for SLE and association with IgG anticardiolipin antibodies. Ann. Rheum. Dis. **59:** 975–981
12 Caponi L., Pegoraro S., Di Bartolo V., Rovero P., Revoltella R. and Bombardieri S. (1995) Autoantibodies directed against ribosomal P proteins: use of multiple antigen peptide as coating agent in ELISA. J. Immunol. Methods **179:** 193–202
13 Van Dam A., Nossent H., Jong J. de, Meilof J., Ter Borg E. J., Swaak T. et al. (1991) Diagnostic value of antibodies against ribosomal phosphoproteins: a cross sectional and longitudinal study. J. Rheumatol. **18:** 1026–1029
14 Ghirardello A., Doria A., Zampieri S. and Gambari P. F. (in press) Diagnostic tests for antiribosomal P protein antibodies: a comparative evaluation of immunoblotting and ELISA assays. J. Autoimmunity
15 Zampieri S., Ghirardello A., Doria A., Tonello M., Bendo R., Rossini K. et al. (2000) The use of Tween 20 in immunoblotting assays for the detection of autoantibodies in connective tissue diseases. J. Immunol. Methods **239:** 1–11
16 Ghirardello A., Doria A., Zampieri S., Gambari P. F. and Todesco S. (2001) Autoantibodies to ribosomal P proteins in systemic lupus erythematosus. Isr. Med. Assoc. J. **3:** 854–857
17 Fabien N., Moreira A., Lavergne J. P., Desbos A., Surgey P., Alves de Olivera C. et al. (1999) Autoantibodies directed against the ribosomal P proteins are not only directed against a common epitope of the P0, P1 and P2 proteins. J. Autoimmunity **13:** 103–110
18 Bonfa E. and Elkon K. B. (1986) Clinical and serological associations of the antiribosomal P protein antibody. Arthritis Rheum. **29:** 981–985
19 Ballesta J. P. G. and Remacha M. (1996) The large ribosomal subunit stalk as a regulatory element of the eukaryotic translational machinery. Progr. Nucleic. Acids Res. Mol. Biol. **55:** 157–193
20 Ballesta J. P. G., Rodriguez-Gabriel M. A., Bou G., Briones E., Zambrano R. and Remacha M. (1999) Phosphorylation of the yeast ribosomal stalk: functional effects and enzymes involved in the process. FEMS Microbiol. Rev. **23:** 537–550
21 Hasler P., Brot N., Weissbach H., Parnassa A. P. and Elkon K. B. (1991) Ribosomal proteins P0, P1 and P2 are phosphorylated by casein kinase II at their conserved carboxyl termini. J. Biol. Chem. **266:** 13815–13820
22 Hasler P., Brot N., Weissbach H., Waleed D., Blount Y., Zhou J. L. et al. (1994) The effect of phosphorylation and site-specific mutations in the immunodominant epitope of the human ribosomal P proteins. Clin. Immunol. Immunopathol. **72:** 273–279
23 Vard C., Guillot D., Bargis P., Lavergne J. P. and Reboud J. P. (1997) A specific role for the phosphorylation of mammalian acidic ribosomal protein P2. J. Biol. Chem. **32:** 20259–20262
24 Zampieri S., Degen W., Ghirardello A., Doria A. and Venrooij W. J. van (2001) Dephosphorylation of autoantigenic ribosomal P proteins during Fas-L induced apoptosis: a possible trigger for the development of the autoimmune response in patients with SLE. Ann. Rheum. Dis. **60:** 72–76
25 Burton D. R. and Barbas C. F. (1994) Human antibodies from combinatorial libraries. Adv. Immunol. **57:** 191–280
26 Winter G., Griffiths A. D., Hawkins R. E. and Hogenboom H. R. (1994) Making antibodies by phage display technology. Annu. Rev. Immunol. **12:** 433–455
27 Tan E., Cohen A. S., Fries J. F., Masi A. T., McShane D. J., Rothfield N. F. et al. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. **25:** 1271–1277
28 Raats J., Bree N. van, Woezik J. van and Pruijn G. (in press) Generating recombinant anti-idiotypic antibodies for the detection of haptens in solution. J. Immunoassay Immunochem.
29 Hoet R. M. A., Raats J. M. H., Wildt R. de, Dumontier H., Muller S., Hoogen F. van den et al. (1998) Human monoclonal antibody fragments from combinatorial antibody libraries directed to the U1snRNP associated U1C protein: epitope mapping, immunolocalization and V-gene usage. Mol. Immunol. **35:** 1045–1055
30 Hoet R. M. A., Pieffers M., Stassen M. H., Raats J. M. H., Wildt R. M. de, Pruijn G. J. M. et al. (1999) The importance of the light chain for the epitope specificity of human anti-U1 small nuclear RNA autoantibodies present in systemic lupus erythematosus patients. J. Immunol. **163:** 3304–3312
31 Kreis T. E. (1986) Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. EMBO J. **5:** 931–941
32 Wildt R. M. de, Finnern R., Ouwehand W. H., Griffiths A. D., Venrooij W. J. van and Hoet R. M. A. (1996) Characterization of human variable domain antibody fragments against the U1 RNA-associated A protein, selected from a synthetic and patient-derived combinatorial V gene library. Eur. J. Immunol. **26:** 629–639
33 Marks J. D., Hoogenboom H. R., Bonnert T. P., McCafferty J., Griffiths A. D. and Winter G. J. (1991) By-passing immunization: human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. **222:** 581–597

34 Degen W. G. J., Pieffers M., Welin-Henriksson E., Hoogen F. H. J. van den, Venrooij W. J. van and Raats J. M. H. (2000) Characterization of recombinant human autoantibody fragments directed toward the autoantigenic U1-70K protein. Eur. J. Immunol. **30:** 3029–3038
35 Schellekens G. A., Jong B. A. W de., Hoogen F. H. J. van den, Putte L. B. A. van de and Venrooij W. J. van (1998) Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J. Clin. Invest. **101:** 273–281
36 Gausepohl H. and Behn C. (2002) Automated synthesis of solid-phase bound peptides. In: Peptide Arrays on Membrane Supports: Synthesis and Applications, pp. 55–68, Koch J. and Mahler M. (eds), Springer, Heidelberg
37 Mahler M., Mierau R. and Blüthner M. (2000) Fine-specificity of the anti-CENP-A B-cell autoimmune response. J. Mol. Med. **78:** 460–467
38 Raats J. M. H., Roeffen W. F., Litjens S., Bulduk I., Mans G., Venrooij W. J. van et al. (2003) Human recombinant autoantibodies specific for the La (SS-B) autoantigen from patient derived phage display combinatorial antibody libraries. Eur. J. Cell Biol. **82:** 131–141
39 Sun K. H., Liu W. T., Tang S. J., Tsai C. Y., Hsieh S. C., Wu T. H. et al. (1996) The expression of acidic ribosomal phosphoproteins on the surface membrane of different tissues in autoimmune and normal mice which are target molecules for anti-double-stranded DNA antibodies. Immunology. **87:** 362–371
40 Sun K. H., Tang S. J., Lin Y. S. and Sun G. K. (2001) Monoclonal antibodies against human ribosomal P proteins penetrate into living cells and cause apoptosis of Jurkat cells in culture. J. Rheumatol. **40:** 750–756
41 Naranda T. and Ballesta J. P. G. (1991) Phosphorylation controls binding of acidic proteins to the ribosome. Proc. Natl. Acad. Sci. USA **88:** 10563–10567
42 Meesters T. M., Hoet M., Hoogen F. H. J. van den, Verheijen R., Habets W. J. and Venrooij W. J. van (1992) Analysis of an immunodominant epitope of topoisomerase I in patients with systemic sclerosis. Mol. Biol. Rep. **16:** 117–123
43 Raben N., Nichols R., Dolhman J., McPhie P., Sridhar V., Hyde C. et al. (1994) A motif in human histidyl-tRNA synthetase which is shared among several aminoacyl-tRNA synthetases is a coiled-coil that is essential for enzymatic activity and contains the major autoantigenic epitope. J. Biol. Chem. **269:** 24277–24283