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ORIGINAL ARTICLE

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A population of autoantibodies against a centromere-associated protein A major epitope motif cross-reacts with related cryptic epitopes on other nuclear autoantigens and on the Epstein-Barr nuclear antigen 1

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Abstract Autoimmune diseases arise from a host's immune response against self-antigens. The triggering events ultimately resulting in such a break of tolerance are largely unknown. It is also not known why certain molecular structures become autoantigenic. The hypothesis has long been proposed that autoimmune diseases arise from molecular mimicry followed by an epitope spreading mechanism. Recently we have shown that the anti-centromere-associated protein A (CENP-A) immune response is directed against an autoantigenic motif, G/A-P-R/S-R-R, that occurs three times in the N-terminal amino acids of CENP-A. In the present study we used mutational analyses with immobilized oligopeptide arrays to identify the amino acids in this motif that are responsible for antibody binding. In particular, we found that surprisingly mimotopes of this motif are present in a vast number of autoantigens and in the Epstein-Barr nuclear antigen 1. With affinity-purified antibodies we show that the antibodies against this motif are polyclonal and cross-react with several autoantigens. However, in these autoantigens this motif often represents a cryptic epitope explaining the obvious conflict between our results and the known high specificity of autoantibodies. The presence of such an ubiquitous structure on autoantigens suggests a novel peptide-driven mechanism for the evolution of autoantibodies.

Keywords Anti-nuclear antibodies · Autoantigen · Synthetic peptides · Cryptic epitopes · Shared epitopes

Abbreviations *ACA:* Anti-centromere antibodies · *CENP:* Centromere-associated protein · *EBNA:* Epstein-Barr nuclear antigen · *EBV:* Epstein-Barr virus · *ELISA:* Enzyme-linked immunosorbent assay · *SDS:* Sodium dodecyl sulfate · *TBS:* Tris-buffered saline

Introduction

Autoimmune diseases arise from a host's immune response against self-antigens. The triggering events ultimately resulting in such a break of tolerance are largely unknown. However, for more than three decades a hypothesis has proposed that autoimmune diseases arise from a process termed molecular mimicry [1]. According to this model, the host's immune response is directed against antigenic structures on infectious agents that resemble structures on self-antigens followed by an epitope-spreading to other self-epitopes. These other self-epitopes may be hidden in the three-dimensional structure of the molecule [2]. To shed more light on the events leading to this break of tolerance a detailed analysis of the antibody-antigen interactions on a molecular level seems to be mandatory for a better understanding of the mechanisms leading to an autoimmune response.

Several target antigens in scleroderma-related diseases are described, the major ones being topoisomerase 1 (Scl 70), the centromere-associated proteins (CENP) A, B, and C, the PM/Scl particle, the RNA-polymerase I complex, and the U3-snoRNP associated protein fibrillarin [3]. CENP-A and CENP-B are considered to be major target proteins for patients' sera which contain anti-centromere antibodies (ACA) [3].

Recently the epitope distribution on CENP-A was determined [4, 5, 6]. The anti-CENP-A immune response was shown to be directed against two antigenic regions in the N-terminal amino acids of CENP-A. Within the two antigenic regions of CENP-A an autoantigenic motif, G/A-P-R/S-R-R, was identified that occurs three times in these two antigenic regions of CENP-A (motif I: GPRRR, amino acids 2–6; motif II: APRRR, amino acids 12–16; and motif III: GPSRR, amino acids 25–29) [6]. This motif was subsequently identified as the prime target of anti-CENP-A autoantibodies. Using affinity-purified antibodies it was shown that motif I and motif III are immunologically more closely related to each other than to motif II.

Based on these observations the present study aimed at providing a closer examination of the amino acids responsible for antibody binding to this motif and at a possible correlation of this motif with other autoantigens.

Material and methods

Human sera and secondary antibodies

Sixteen ACA-positive sera were selected by enzyme-linked immunosorbent assay (ELISA) screening using recombinant CENP-B and by indirect immunofluorescence on HEp-2 cells (Euroimmun, Lübeck, Germany). Sera were also screened for the presence of antibodies against Epstein-Barr virus (EBV) by Epstein-Barr nuclear antigen (EBNA) 1 ELISA and by EBV western blot (Euroimmun). All sera were tested in commercial ELISA and western blotting systems for the presence of antibodies against U1-snRNP, Sm, SS-a, SS-B, Scl-70, Jo-1, histones, dsDNA, ssDNA, and ribosomal P proteins, and subsequently shown to be negative for those antibodies (Euroimmun). Demographic, clinical, and serological data are given in Table 1. Normal sera from healthy donors were

Table 1 Clinical, serological, and demographic data of patient and control sera (*n.d.* not determined)

Serum sample[a]	Years from disease onset to serum sampling	Diagnosis	ACA titer IIF	ANA (western blot) CENP-		
				A	**B**	**C**
Patient sera						
CENP-0001	9	Systemic sclerosis, limited	1/32,000	+	+	+
CENP-0002	10	Systemic sclerosis, limited	1/10,000	+	–	–
CENP-0003	10	Undiff. connect. tissue disease	1/10,000	–	–	+
CENP-0005	14	Rheumatoid arthritis, vasculitis	1/3,200	+	+	–
CENP-0006	8	Systemic sclerosis, limited	n.d.	+	+	–
CENP-0007	22	Systemic sclerosis, limited	1/32,000	+	+	–
CENP-0008	4	Systemic sclerosis, limited	n.d.	+	+	–
CENP-0009	4	Undiff. connect. tissue disease	1/10,000	–	–[b]	–
CENP-0010	14	Systemic sclerosis, limited	1/32,000	+	+	+
CENP-0011	16	Systemic sclerosis, limited	n.d.	+	+	+
CENP-0012	2	Systemic sclerosis, limited	1/32,000	+	+	–
CENP-0014	1	Undiff. connect. tissue disease	1/32,000	+	–	+
CENP-0017	5	Systemic sclerosis, limited	1/10,000	(+)	+	+
CENP-0018	1	Systemic sclerosis, limited	1/10,000	(+)	+	–
CENP-0019	4	Systemic sclerosis, limited	1/10,000	+	+	+
CENP-0021	2	Systemic sclerosis, limited	n.d.	+	+	+
Negative human control sera						
NM1	–	Control sample	–	–	–	–
NMB1	–	Control sample	–	–	–	–
NDM1	–	Control sample	–	–	–	–
N336	–	Control sample	–	–	–	–
N338	–	Control sample	–	–	–	–
NUW1	–	Control sample	–	–	–	–

[a] All sera were positive for anti-EBV antibodies either by ELISA with recombinant EBNA-1 or by western blot with EBV proteins
[b] Serum CENP-0009 was positive by ELISA with recombinant CENP-B antigen (Euroimmun, Lübeck, Germany)

obtained from Dr. H.J. Lakomek, Medizinische Klinik C, Universitätsklinik Düsseldorf, and from volunteers from our laboratory. Secondary antibodies (alkaline phosphatase-coupled goat anti-human IgG, horseradish peroxidase coupled goat anti-human IgG) were purchased from Dianova, Germany.

Immobilized oligopeptides and subsequent immunoassay

Peptides were synthesized with an ASP222 machine on activated membranes (Abimed, Germany) using fMoc protected amino acid derivatives (SPOT system, Genosys, UK) according to the procedure described by R. Frank [7]. Following completion of the synthesis membranes were washed three times in Tris-buffered saline: 10 mM Tris/Cl pH 7.6, 150 mM NaCl (TBS) for 10 min per wash. Nonspecific binding sites were subsequently blocked by overnight incubation of the membranes in blocking buffer containing TBS/0.2% Tween, 50% inactivated horse donor serum, 5% sucrose (w/v), and 1× blocking buffer (Genosys) at room temperature. Unbound blocking proteins were removed by a single washing-step in TBS/0.2% Tween 20 followed by incubation for 2 h with serum dilutions of 1/100 in antibody buffer [TBS containing 3% horse serum, 1× blocking buffer, 5% sucrose (w/v), 0.05% Tween 20 (v/v)]. The membranes were washed three times in TBS/0.2% Tween-20 and three times in TBS for 5 min per wash. For visualization of bound antibodies we used horseradish peroxidase coupled goat anti-human IgG antibodies at a dilution of 1/5000 in antibody buffer with an incubation time of 75 min. Membranes were washed as described. Detection was performed with enhanced chemiluminescence western blotting detection reagents (Amersham International, UK).

Antigen preparation and immunoblot

Nuclear extracts were prepared as previously described [8]. In brief, 1×10^9 HeLa S3 cells were washed in PBS and in 30 ml buffer A (15 mM Tris/Cl, pH 7.4; 80 mM KCl; 2 mM EDTA; 0.2 mM EGTA; 0.2 mM spermine; 0.5 mM spermidine; 7 mM 2-mercaptoethanol). Washed cells were homogenized in 30 ml buffer B (buffer A + 0.1% digitonin; 0.1 mM phenylmethylsulfonyl fluoride; 1 µM Leupeptin; 1 µM Pepstatin A) and nuclei were collected by centrifugation at 750 *g*, 4°C. Nuclei were resuspended in 10 ml 2× sodium dodecyl sulfate (SDS) centromere sample buffer [20 mM Tris/Cl pH 6.8; 20%(v/v) glycerol; 10%(v/v) 2-mercaptoethanol; 6% SDS; 0.02% bromophenol blue; 0.1% digitonin; 0.1 mM phenylmethylsulfonyl fluoride; 1 µM Leupeptin; 1 µM Pepstatin A) and sonicated 5×5 s at 45 W. For 15% SDS polyacrylamide gel electrophoresis the equivalent of 1×10^6 cells was loaded per slot. Following the gel run proteins were electrotransferred onto nitrocellulose (Schleicher& Schüll, Germany; 0.4 µm pore size) overnight at 4°C according to common procedures. Antigens were detected with serum dilutions of 1/100 followed by secondary antibody (goat anti-human IgG coupled to alkaline phosphatase) in conjunction with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

Affinity-purification of antibodies from polyclonal sera

Thirty identical peptides were synthesized on activated membranes, blocked, washed, and incubated with serum (1/50) as described. Nonspecific antibodies were removed by washing the membranes three times in TBS/0.2% Tween-20 and three times in TBS for 5 min per wash. Specifically bound antibodies were eluted with 1.5 ml elution buffer (150 mM NaCl, 5 mM Glycin, 0.1% Tween 20, pH 2.4) and neutralized with 0.6 ml neutralization buffer (1 M Tris/Cl, pH 8.0) as described [9]. Affinity-purified antibodies were concentrated using centricon-30 microconcentrators at 3750 rpm and 4°C to a final volume of 50 µl.

Computer-assisted homology search

A search for structural homologies was performed at the SWISS-MODEL Automated Comparative Protein Modelling Server (http://www.expasy.ch/swissmod/SWISS-MODEL.html). The program BESTFIT from the HUSAR program package (version 5.0) of the German Cancer Research Center, Heidelberg, Germany, was used to scan known autoantigen sequences for the presence of a stretch of amino acids homologous to the sequence GPXRR.

Results

Mutational analyses of the epitope motif G/A-P-R/S-R-R identify mimotopes of this motif

In a recent study we identified the epitope motif G/A-P-R/S-R-R as the prime target sequence of anti-CENP-A autoantibodies. In order to analyze the immune response against this epitope motif in more detail the sequence GPRRR was subjected to a mutational analysis by successively replacing each amino acid in the pentameric peptides GPRRR by each of the 20 naturally occurring amino acids. The resulting peptides were probed with several anti-centromere sera (CEN-0006, CEN-0017, and CEN-0018; Fig. 1A). This showed that glycine at position 1 and proline at position 2 of the motif are essential for antibody binding since neither amino acid can be replaced by any other amino acid without completely inhibiting antibody binding. In addition, since the arginine at position 4 can be replaced only by alanine, this position of the epitope motif is crucial for antibody binding of the sera CEN-0006, and CEN-0018. With regard to serum CEN-0017 this position is more flexible. In contrast, with regard to sera CEN-0006, and CEN-0018 the amino acid positions within the epitope motif that are apparently most flexible are positions 3 and 5. Therefore in a second approach we generated a dipeptide library based on the amino acid sequence GPx_1Rx_2 with x_1 and x_2 representing any of the 20 naturally occurring amino acids (Fig. 1B). The library then was probed with the anti-centromere serum CEN-0018. The results confirm the immunological flexibility of positions 3 and 5 within the epitope motif, because most of the possible amino acid combinations are tolerated at these positions without significantly altering the immunoreactivity (Fig. 1B). Some sequences gave even stronger signals than the wild-type sequences (e.g., GPTRA or GPSRQ vs. GPRRR or GPSRR), suggesting higher affinities of the antibodies to these peptides.

Homology searches suggest the presence of the epitope motif in other autoantigens and in the EBNA-1 protein

A homology search in the SWISSMODEL three-dimensional database with the template sequence GPRRRSRKPEAPRRRSPSPTPTPGPSRR which spans amino acids 2–29 of the CENP-A amino acid sequence, and which contains all three epitopes related to the motif G/A-P-R/S-R-R showed that this sequence has some structural

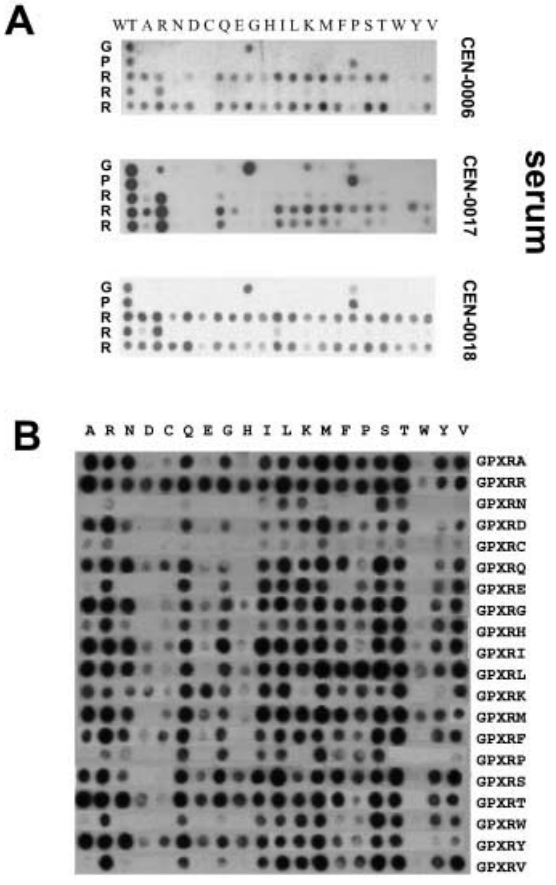

Fig. 1A,B Mutational analysis of the epitope GPRRR. In order to identify the amino acids necessary for antibody binding the CENP-A epitope 3 was subjected to mutational analyses. **A** Every amino acid of the 5-mer peptide GPRRR was successively replaced by all 20 naturally occurring amino acids. Probing of the resulting peptides with several sera (CEN-0006, CEN-0017, and CEN-0018) revealed that glycine, proline, and arginine at positions 1, 2, and 4, respectively, are essential for binding of the corresponding antibodies. Serine and arginine at positions 3 and 5, respectively, do not contribute significantly to antibody binding as far as sera CEN-0006 and CEN-0018 are concerned. For these two sera the amino acids at positions 3 and 5 can be replaced by almost any of the 20 naturally occurring amino acids. As for serum CEN-0017 position 4 is more flexible. **B** To further demonstrate the flexibility of the epitope GPRRR, serine and arginine at positions 3 and 5 were mutated simultaneously to create a dipeptide library. The library was probed with serum CEN-0018. The results demonstrate the existence of a vast number of mimotopes, some of which react even more strongly than the wild-type peptide (compare e.g., GPRRR vs. GPTRA or GPSRQ) suggesting a higher affinity of the antibodies to those peptides

homology to the EBV-related protein EBNA-1 (accession number 1VHI; data not shown).

These findings of immunological flexibility prompted us to search known autoantigen amino acid sequences and the amino acid sequence of EBNA-1 for the presence of peptides homologous to the motif G/A-P-R/S-R-R using the program BESTFIT from the HUSAR program package. Altogether, 52 amino acid stretches within known autoantigens were found to be more or less homologous to the motif GPX_1RX_2. Of those 52 amino acid stretches 23 were found to have three or more residues identical to the motif GPX_1RX_2 (group 1, Table 2), 10 had two identical and at least one similar residues (group 2), 16 had two identical residues (group 3), and three had 1 identical and three similar residues (group 4). From the SWISSPROT database nine control proteins were randomly selected and analyzed in the same way as the autoantigen sequences. The negative control proteins were selected based on their presence in the nucleus, to the best of our knowledge the absence of autoantigenic reactivity, and the presence of a peptide stretch that is to some degree homologous to the query sequence. In these control proteins the relevant peptide stretches belonged to groups 1, 2, and 3 as indicated (Table 2, negative control peptides).

Anti-centromere sera contain antibodies reactive with related peptides from other autoantigens

Based on our findings we selected 11 candidate sequences for further analysis [EBNA-1 (motif 1), CENP-A (epitope 1), CENP-A (epitope 2), CENP-A (epitope 3), CENP-B, CENP-C, Ku-80, Ro-52, U1-RNP-C, Sm B/B′, Sm D1, all from homology group 1]. These 5-mer candidate sequences were synthesized within their amino acid context thus yielding 10-mer peptides with the motif itself being located in the middle of the respective peptide. The CENP-A epitope 1 and the CENP-B peptide are located at the N-termini, and the Sm D1 peptide is located at the C-terminus of the respective antigens. Therefore in these peptides the 5-mer sequences were not located in the center of the corresponding 10-mer peptides. The 11 peptides were probed with selected highly reactive anti-centromere sera (CEN-0006, CEN-0008, CEN-0014, CEN-0017, CEN-0018, and CEN-0021) and a normal human control serum (NHS 1) (Fig. 2). As expected, all anti-centromere sera tested reacted with the three CENP-A motifs.

In addition, all anti-centromere sera reacted with the CENP-B motif, the CENP-C motif, the Sm D1 motif, and the EBNA-1 motif 1. Two sera (CEN-0006, CEN-0018) reacted strongly and three sera (CEN-0008, CEN-0017, CEN-0021) reacted weakly with the Ku 80 motif. Two sera (CEN-0006, CEN-0018) reacted clearly with the Sm BB' motif. One serum (CEN-0014) reacted weakly with the U1-snRNP C motif. These findings prompted us to scan more autoantigen-derived peptides containing the sequences homologous to the motif GPX_1RX_2 and the negative control peptides using more anti-centromere sera. Altogether 26 different autoantigen-derived peptides and 9 negative control peptides were probed with 16 anti-centromere sera and 6 normal human control sera (Table 3). The number of autoantigen-derived peptides recognized by the individual sera ranged from 4 of 26 to 18 of 26. The intensities of the immunoreactivities were assessed visually. The results show that for a given serum the intensities of immunoreactivities with different peptides vary significantly. No correlation was found be-

Table 2 Sequence-comparision (Bestfit) of the query peptide GPXRR to known autoantigens

Peptide number	Antigen	Accession number	Bestfit result[a]	Amino acid position[b]	Homology group[c]
1	CENP-A (epit. 1)	P49450	/////m**GPRRR**srkp[d]	2	1
2	CENP-A (epit. 2)	P49450	///kpe**APRRR**sp	12	1
3	CENP-A (epit. 3)	P49450	///ptp**GPSRR**gp	25	1
4	CENP-B	S18735	/////m**GPKRR**qltf[d]	2	1
5	CENP-C	Q03188	///ees**GPSR**Lnn	685	1
6	HMG-2	P26583	///gkk**GPGR**Ptg	173	1
7	HMG-14	P05114	///akeE**PKRR**sa	15	1
8	HMG-17	P05204	///vkdE**PQRR**sa	19	1
9	Ku 80	J04977	///rgd**GPFR**Lgg	181	1
10	La	P05455	///gat**GPVKR**ar	380	1
11	Ro 52	U01882	///aft**GPLR**Pff	340	1
12	U1 70 k	M22636	///evy**GPIKR**ih	127	1
13	U1 C	X12517	///slp**GPPR**Pgm	85	1
14	Sm B/B’	P14678	///gla**GPVR**Gvg	129	1
15	Sm B3	J04615	///gla**GPVR**Gvg	129	1
16	Sm D1	P13641	rgrgrg**GPRR**[d]	116	1
17	tRNA PL12	P49588	///fidE**PRRR**pi	596	1
18	tRNA PL7	M63180	///lcr**GPHVR**ht	251	1
19	E1-β	P21953	///gae**GHWRR**lp	21	1
20	E2	P10515	///llgS**PGRR**yy	47	1
21	EBNA1 (epit. 1)	S42440	///ggs**GPQRR**gg	30	1
22	EBNA1 (epit. 2)	S42440	///sgs**GPRHR**dg	63	1
23	EBNA1 (epit. 3)	S42440	///pqp**GPLR**Esi	552[e]	1
24	Coilin	S50113	///isd**GPSK**Vtl	260	2
25	HMG-1	P09429	///dpnA**PKR**Pps	93	2
26	NOR 90	P17480	///klr**GP**NPKss	661	2
27	PM/Scl-75	JH0446	///pskK**PVKR**rk	344	2
28	Ra 33	P22626	///sny**GPMK**Sgn	314	2
29	Scl 70	P11387	///ediK**PLKR**pr	134	2
30	SP 100	P23497	///twr**IPSRK**rr	443	2
31	rRNP P0	M17885	///tgl**GPEK**Tsf	131	2
32	cANCA	P24158	///ctfVPRRKag	192	2
33	Histone H4	X83548	///lgk**GGAKR**hr	14	2
34	Fibrillarin	P22087	///div**GP**DGLvy	182	3
35	HP1-β	P23197	///rglE**PER**Iig	119	3
36	Ku 70	J04611	///cryT**PRR**Nip	401	3
37	PM-Scl 100	JH0796	///kks**GP**LPSae	593	3
38	Ro 60	P10155	///tiaD**PDDR**gm	510	3
39	U1A	X06347	///avq**GP**VPGmp	139	3
40	Sm D2	P43330	///fnt**GP**LSVlt	27	3
41	rRNP P1	M17886	///gag**GP**APAag	67	3
42	tRNA EJ	P41250	///ydf**GP**VGCal	97	3
43	tRNA Jo1	Z11518	///aql**GP**DESkq	46	3
44	tRNA OJ	U04953	///fyd**GP**PFAtg	46	3
45	pANCA	P05164	///vdl**GP**CWAgg	16	3
46	E1-α	P12694	///aar**GP**GYGim	288	3
47	Histone H1	X57130	///kpa**GP**SVSel	40	3
48	Histone H2a	X00089	///kqg**GKAR**Aka	9	3
49	Histone H3	X00090	///irk**LPFQR**lv	66	3
50	PM-Scl 75	JH0446	///qek**APSKK**pv	339	4
51	Sm D3	P43331	///sig**VPIK**Vlh	5	4
52	Histone H2b	X00088	///aks**APKKG**sk	8	4
Control peptides[f]					
53	Bcl 2	P10415	///ely**GPSMR**pl	203	1
54	Nucleoplasmin 3	O75607	///sgs**GPVRI**tg	129	1
55	Bub 3	O43684	///thd**APIRC**ve	97	2
56	Bub 1	O43683	///arm**GPSVG**sq	326	3
57	chk 1	O14751	///ssq**NPWQR**lv	368	3
58	Cyclin f	P41002	///edk**GPQDP**qa	664	3
59	DNase I	P24855	///vvs**EPLGR**ns	91	3
60	Hox A1	P49639	///ssc**GPSYG**sq	96	3
61	TAF II68	Q92804	///dgr**GPMTG**ss	196	3

[a] Amino acid context is given in lower-case letters, the motif itself in upper-case letters and in bold

[b] Numbering refers to the first amino acid of the motif

[c] Results are grouped as follows: group 1=at least 3 identical residues, group 2=2 identical and at least 1 similar residue, group 3=2 identical residues, group 4=fewer than 2 identical or similar residues

[d] Peptides are located at the N-terminus (CENP-A, epitope 1, CENP-B) or at the very C-terminus (Sm D1) of the corresponding antigen

[e] This represents amino acid 92 in the experimentally determined three-dimensional structure (accession number 1B3TA/1B3 TB)

[f] Control proteins were randomly chosen as common nuclear proteins not known as autoantigens

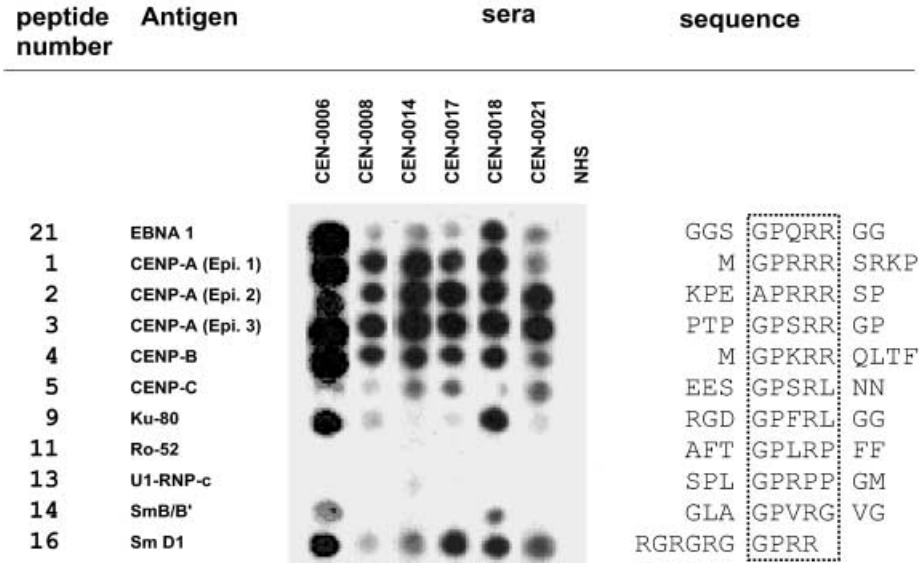

Fig. 2 Immunoreactivity of the epitope motif GPX_1RX_2 in other autoantigens. Peptides with sequence homologies to the epitope motif GPX_1RX_2 were synthesized as 10-mers as indicated and probed with anti-centromere autoantisera. The peptides are numbered according to Table 3. The results clearly demonstrate the presence of cross-reactive antibodies against this motif in the sera tested. All anti-centromere sera react with the corresponding epitopes on the three centromere antigens and on the EBNA-1. Most sera also react with the epitopes on the Ku-80 and on the Sm D1 antigen. Two sera react with epitopes on the Sm B/B′ antigen (CEN-0006, and CEN-0018) and one serum reacts weakly with the epitope on the U1-RNP-C antigen (CEN-0014)

tween the number of peptides recognized and the clinical data of the patients. As for the negative control peptides only two sera reacted weakly with 1 of 9, or 2 of 9. No reactivity at all was observed with the normal human control sera.

Affinity-purified antibodies against the epitope motif G/A-P-R/S-R-R cross-react with related epitopes on other autoantigens

To assure that our results are based on true cross-reactivities, antibodies affinity-purified from the epitope-containing peptides were analyzed by western blotting on cellular extracts and by peptide assays (Fig. 3). Nitrocellulose strips with cellular extracts were prepared as described in the materials and methods section (Fig. 3A), and 11 peptides were synthesized on membranes as described above (Fig. 3B). Both the nitrocellulose strips with cellular extracts and the peptides were incubated with the serum CEN-0006. Antibodies against the epitope motif in the serum CEN-0006 were affinity-purified from the EBNA-1 epitope 1 (Fig. 3, lanes 2), from the CENP-A epitope 1 (lanes 3), from the CENP-A epitope 2 (lanes 4), and from the CENP-A epitope 3 (lanes 5), as described above, and reincubated on the nitrocellulose strips with cellular extracts (Fig. 3A) or the peptides (Fig. 3B). One nitrocellulose strip with cellular extracts and one set of peptides were incubated with a normal human control serum (N338; lanes 6).

All affinity-purified antibodies reacted with the cellular CENP-A and all but antibodies affinity-purified from the CENP-A epitope 2 reacted, although weakly with CENP-B (Fig. 3A). However, antibodies affinity-purified from the CENP-A epitope 2 reacted only weakly with the cellular CENP-A (Fig. 3B, lane 4). The pattern of peptide recognition was more diverse. Antibodies affinity-purified from the EBNA-1 epitope 1 clearly reacted with the same peptides as the serum CEN-0006 with the exception of the CENP-A epitope 2 which was recognized by serum CEN-0006 but was not recognized by the antibodies affinity-purified from the EBNA-1 epitope 1 (Fig. 3B, lanes 1, 2). The intensity of the immune reaction with the peptides 10 (SmB/B′) and 11 (Sm D1) was inverted when the total serum response was visually compared with the response of the antibodies affinity-purified from the EBNA-1 epitope 1. When compared with each other, antibodies affinity-purified from either the CENP-A epitopes 1 or 3 reacted in the same manner in that both antibodies recognized the same peptides (EBNA-1, CENP-A epitope 1, CENP-A epitope 3, CENP-B, Ku-80) but recognized neither the CENP-A epitope 2 nor the Sm B/B′ epitope nor the Sm D1 epitope. Antibodies affinity-purified from the CENP-A epitope 2 reacted exclusively with this epitope. Comparable results were obtained with serum CEN-0018 (data not shown).

Mimotopes of the epitope motif G/A-P-R/S-R-R present on other autoantigens often represent cryptic epitopes

Since in almost all cases the initial immunofluorescence studies showed a clear centromere staining without apparent cospecificity of the serum antibodies, and since no serum displayed other known specificities in the com-

Table 3 Immunoreactivity of anti-centromere autoantibodies with peptides related to the G/A-P-R/S-R-R motif

Peptide number[a]	Serum/intensity[b] CEN-																
	0001	0002	0003	0005	0006	0007	0008	0009	0010	0011	0012	0014	0017	0018	0019	0021	NHS[c]
1	++	+	+	++	+++	++	+++	++	++	++	++	+++	+++	+++	+	+	–
2	–	+++	–	–	++	+++	+++	+	++	++	–	+++	+++	+++	–	+++	–
3	+++	+++	++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–
4	++	++	–	++	+++	+++	+++	–	+	+	++	+++	+++	+++	–	+	–
5	+	–	–	–	–	–	+	+	–	+++	+	++	++	+	–	++	–
7	+	–	+	–	–	++	++	+	–	+	+	+	++	–	+	++	–
8	+	–	+	–	–	+	+	++	+	+	+	–	–	–	+	–	
9	–	+	+	–	+++	++	+	+	+	++	+	–	+	+++	–	+	–
10	+	–	–	–	–	–	+	–	–	+	+	+	+++	–	+	++	–
11	–	–	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–
12	–	–	–	–	–	++	–	–	–	–	–	–	–	–	–	–	–
13	–	–	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–
14	–	–	+	–	++	–	–	+	+	+	+	–	–	++	–	–	–
16	+	–	–	–	+++	+	+	+	–	+	+	++	+++	+++	–	+++	–
17	–	–	–	–	–	–	+	–	–	–	+	+	++	–	–	+	–
19	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
21	+	+	+	+	+++	+	+	+	+	+	+	+	+	+++	–	+	–
24	–	–	–	+	+	–	–	–	–	–	–	–	+	+	–	–	–
25	+	–	+	–	–	+	+	–	–	+	++	+	+++	–	–	+	–
30	+	–	–	–	–	–	+	–	–	+	+	+	+++	–	–	++	–
31	–	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–
32	–	–	–	–	–	–	+	–	–	–	–	+	+	–	–	++	–
36	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	+	–
39	+	–	–	–	–	–	–	–	–	++	+	–	+	+	–	–	–
40	+	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–
Number of peptides recognized	13	6	10	6	10	11	15	12	9	18	18	14	17	13	4	16	–
Negative control peptides																	
53	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
54	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
55	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
56	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
57	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
58	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
59	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
61	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Number of peptides recognized	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	2	–

[a] Numbering refers to Table 2. Peptides were synthesized as 10-mers as indicated in Table 2
[b] Intensity of the reaction was assessed visually: +++ = very strong reactivity, ++ = clear reactivity, + = weak reaktivity, – = no reactivity
[c] NHS is a "virtual serum" representing the results from all normal human control sera tested

Table 4 Reactivity of selected anti-centromer sera with peptides of different lengths (*n.d.* not determined)

Pepitde		Sequence	CEN-					
Epitope	Amino acid position		0005	0006	0014	0017	0018	0019
CENP-A (3)	25–29	//////**GPSRR**	–	+	–	+	+	+
		///PTP**GPSRR**GP	+	+	+	+	+	+
		SPTPTP**GPSRR**GPSL	+	+	+	+	+	+
CENP-B	2–6	**/GPKRR**	n.d.	+	n.d.	+	+	n.d.
		M**GPKRR**QLTF	+	+	+	+	+	–
		M**GPKRR**QLTFREKSR	+	–	+	+	–	–
EBNA-1 (1)	27–36	/////**GPQRR**	n.d.	+	n.d.	+	+	n.d.
		//GGS**GPQRR**GG	+	+	+	+	+	–
		GSGGS**GPQRR**GGDNH	–	–	–	–	–	–

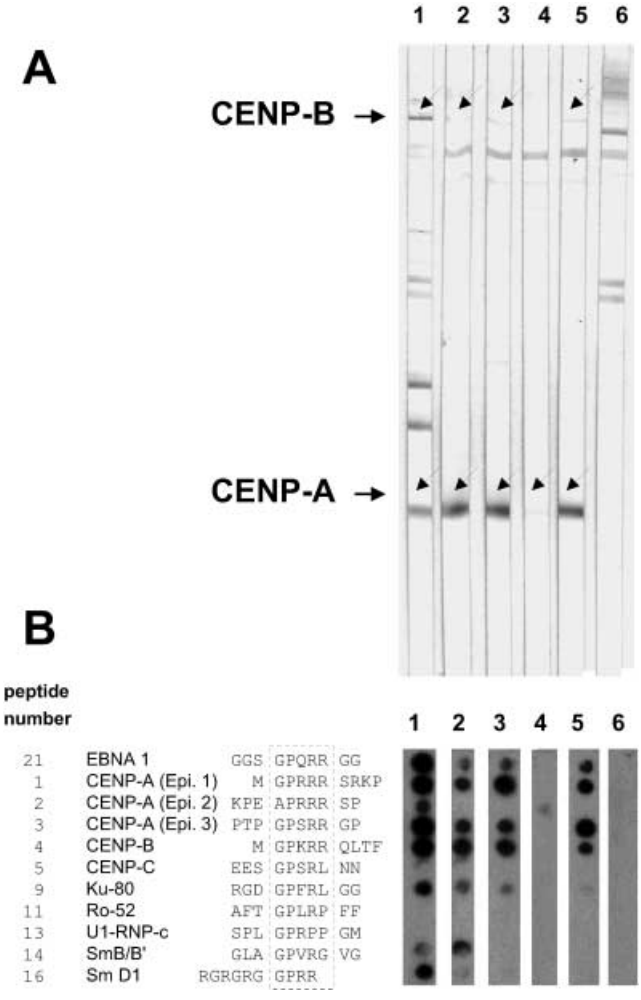

Fig. 3A,B Cross-reactivity of antibodies against the epitope motif GPX_1RX_2. Antibodies in the patient's serum CEN-0006 against the epitope motif GPX_1RX_2 were affinity-purified from the corresponding peptides in EBNA-1 (epitope 1, GGSGPQRRGG, positions 27–36, *lanes 2*), CENP-A (epitope 1, MGPRRRSRKP, positions 1–10, *lanes 3*), CENP-A (epitope 2, KPEAPRRRSP, positions 9–18, *lanes 4*), CENP-A (epitope 3, PTPGPSRRGP, positions 22–31, *lanes 5*) and reincubated on western blots with cellular extracts from HeLa S3 cells (**A**) or on immobilized peptides as indicated (**B**). The corresponding patient's serum CEN-0006 (*lane 1*) and the normal human control serum N338 (*lane 6*) served as controls. Antibodies affinity-purified from the epitope 1 of EBNA-1, and the epitopes 1 and 3 of CENP-A crossreact with cellular CENP-A and CENP-B (**A**, *lanes 2, 3, 5*) and with peptides from EBNA-1, CENP-A (epitopes 1, 3), CENP-B, CENP-C, and Ku-80 (**B**, *lanes 2, 3, 5*). Antibodies affinity-purified from the EBNA-1 epitope 1 additionally cross-react with the peptides from Sm B/B′, and Sm D1 (**B**, *lane 2*). Antibodies affinity-purified from the CENP-A epitope 2 react only with cellular CENP-A (**A**, *lane 4*) and with the peptide from CENP-A epitope 2 (**B**, *lane 4*). **B** Peptides numbered according to Table 3

mercial assay systems, the presence of cryptic epitopes on other autoantigens was assumed. Indeed, when in a representative experiment the peptides bearing the relevant epitopes of the CENP-B autoantigen and of the EBNA-1 antigen were synthesized in different lengths and then probed with several anti-centromere sera, the CENP-A epitope 3 was recognized regardless of the number of surrounding amino acids with the exception of sera CEN-0005 and CEN-0014, which did not recognize the 5-mer peptide. In contrast, the CENP-B epitope and the EBNA-1 epitope were recognized in most cases only as 10-mer or 5-mer peptides (Table 4). These results indicate that the relevant epitopes of those two antigens may represent cryptic epitopes that are hidden in the three-dimensional structures.

Discussion

Mutational analyses using synthetic peptides have been used to identify the amino acids in an epitope that contribute to the binding of their cognate antibodies and to identify mimotopes that are recognized by cross-reactive antibodies [10, 11]. In a previous study we identified the motif G/A-P-R/S-R-R which occurs three times within the N-terminus of CENP-A as the prime target of the anti-CENP-A autoimmune response [6]. The results of our mutational analyses suggest that the amino acid sequence GPx_1Rx_2 represents the consensus sequence within this motif when the motif is synthesized as a 5-mer peptide (Fig. 1). Of particular relevance in this motif are glycine at position 1 and proline at position 2. The results also explain our previous finding of some cross-reactivity between anti-epitope 1 antibodies and anti-epitope 3 antibodies, but not anti-epitope 2 antibodies [6]. Muro et al. [5] found no such cross-reactivity since in their study the important glycine at position 2 of the CENP-A amino acid sequence was missing. Our results also confirm that at least two populations of antibodies are involved in the anti-CENP-A immune recognition of this motif [6]. One population is directed against epitopes 1 and 3, and one is directed against epitope 2.

The most interesting finding, however, is the presence of homologous amino acid sequences in other known autoantigens and in the sequence of EBNA-1. These homologies suggest that this sequence represents a common motif in autoantigens. The homologous sequences were grouped according to the degree of homology to the query sequence (Table 2). Upon probing of representative peptides with anti-centromere sera it became evident that the immunoreactivity of the peptides is not directly correlated with the degree of sequence homology (Tables 2, 3). Thus our results reflect rather an immunological or structural relationship than a plain similarity by sequence homology. Since the peptides on a given spot are in 10,000-fold molar excess over the bound antibodies, the varying intensities of immunoreactivities can be attributed to the different binding affinities of the antibodies to the respective peptides [12]. In addition, recognition of the relevant epitopes may depend on the lengths of the peptides targeted by the antibodies suggesting the presence of cryptic epitopes. Such cryptic epitopes have already been demonstrated several times [2, 13].

Several studies have been reported that antibodies affinity-purified from CENP-B are cross-reactive with CENP-A and vice versa [14]. In the case in which such

cross-reactivity was not detected, N-terminally truncated CENP-B proteins were used, excluding the N-terminal epitope GPKRR [15]. The identification of the common epitope motif on both the CENP-A and the CENP-B autoantigen may explain these findings. In this context it is of interest that the C-terminus of the SmD1 antigen which contains the above epitope motif has already been identified as a major target of anti-SmD1 autoantibodies [16].

The novel finding of the presence of a common epitope motif on several autoantigens raises the possibility that, starting from such a common "ancestral" epitope, the autoimmune response extends – via epitope spreading followed by diversification and affinity maturation mechanisms of the corresponding B-cells – to other autoantigens and results in a polyclonal immune response against the relevant peptides. An epitope-spreading mechanism has been demonstrated recently for the development of autoantibodies against the Sm B/B′ antigen [17]. Furthermore, it was reported that anti-centromere autoantibodies frequently accompany antibodies directed against other autoantigens [18]. The polyclonality of an autoimmune response against autoantigens was been suggested more than a decade ago [3]. Based on our results this model seems to apply even at the level of distinct epitopes. In this context two recent publications are of interest [19, 20]. These studies showed that immunization of mice with both autologous or xenogenic peptides derived from the autoantigen Ro60 results in a break of tolerance against this antigen. Interestingly, as the result of an epitope-spreading mechanism autoantibodies to other autoantigens were observed which are physically not related to the Ro60-containing RNP particle [19, 20]. In addition, it was suggested that some of those antibodies were directed against cryptic epitopes [20].

In a recent study we proposed that the major epitope on the PM/Scl-100 autoantigen resembles a structure on another autoantigen, the heterochromatin binding protein HP-1β [11]. That finding together with our data presented here and the findings of Deshmukh et al. [19, 20] suggests that many autoantigens are immunologically and structurally related, although such structures may be hidden in native antigens. Such an immunological or structural relationship may be the result of a novel peptide-driven mechanism for the evolution of autoantibodies.

The role of antibodies against EBV-related proteins, in particular EBNA-1, must at present remain unclear. It has been proposed several times that EBV is involved in the triggering of autoimmune diseases. However, there is still no direct proof for this [21].

The apparent contradiction to the hitherto known high specificity of autoantibodies may be explained by the fact that for diagnostic purposes most test systems utilize proteins rather than peptides to detect as many epitopes as possible. Our assays, in contrast, use peptides that are taken out of their amino acid context and thus are able to expose cryptic epitopes. A second possibility is that the epitopes are posttranslationally modified in most autoantigens but not in the CENP-A autoantigen, as has been reported recently for Sm-D – and for filaggrin-related epitopes [22, 23].

In summary, we have identified a common amino acid sequence motif on autoantigens and on the EBNA-1 that is recognized by a population of autoantibodies present in anti-centromere sera. Future studies should be designed to investigate this phenomenon in detail.

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