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Anti-Ro fine specificity defined by multiple antigenic peptides identifies components of tertiary epitopes

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SUMMARY

Anti-Ro (or SSA) is a clinically important autoantibody that is found in 25–40% of patients with systemic lupus erythematosus as well as an even greater proportion of patients with Sjögren's syndrome or subacute cutaneous lupus. We have studied the binding of anti-Ro sera to multiple antigenic peptides constructed from the sequence of the 60-kD Ro molecule. The results demonstrate that sera bind these peptides in solid-phase assay. Surprisingly, some of these peptides also form a precipitin line in double immunodiffusion with anti-Ro sera. Formation of lines of identity in double immunodiffusion as well as absorption studies indicate that peptides distant in the primary amino acid sequence and without shared sequence are bound by the same antibody. In addition, data from surface plasmon resonance demonstrate that peptides identified in this manner have protein–protein interactions. Thus, these techniques may identify the components of conformational epitopes.

Keywords systemic lupus erythematosus autoantigen autoantibody peptides epitopes

INTRODUCTION

Anti-Ro (or SSA) is present in the sera of 25–40% of patients with systemic lupus erythematosus (SLE), as well as most patients with Sjögren's syndrome (SS), subacute cutaneous lupus, and mothers of infants with congenital lupus [1]. Anti-Ro is associated with several clinical features of SLE [2], including the presence of anti-La, which is rarely if ever found without anti-Ro [3].

The major antigen bound by anti-Ro sera is a 60 000 mol. wt protein that is non-covalently associated with one of several hY RNAs. The La antigen is also associated, at least at times, with the Ro ribonucleoprotein complex. A 52-kD protein is bound in immunoblot by some sera with anti-Ro [4], but the physical relationship of this protein with La and 60-kD Ro is controversial [5,6].

The 60-kD Ro protein loses much of its antigenicity after being denatured [7]. Despite this, we and others have found that most sera with anti-Ro bind multiple groups of overlapping short peptides from 60-kD Ro [8–10]. Recent data have demonstrated that antibody binding the peptide epitopes also binds the native molecule [11]. These data suggest that antibody binding the short peptide linear epitopes of 60-kD Ro is also part of the immune response to the native molecule.

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Multiple antigenic peptides (MAP) have several copies of a short peptide covalently bound to a branching lysine backbone, and have been used for a variety of purposes, including immunization and immunoassay [12–14]. We have produced the previously defined 21 peptide epitopes of 60-kD Ro as MAP and used them to define the peptide epitopes in a large cohort of anti-Ro-positive SLE patients. We find that these constructs can be used to identify epitopes bound by anti-Ro sera using a solid-phase assay. Furthermore, Ro-MAP and patient sera produce precipitin lines in double immunodiffusion. Data using immunodiffusion and affinity chromatography indicate that specific sets of peptide epitopes are bound by the same subset of anti-Ro antibodies. Using surface plasmon resonance we find protein–protein interactions among peptides identified in this way. These data imply that the identified peptide epitopes are component parts of conformational antibody determinants.

PATIENTS AND METHODS

Patients and sera

Patients studied had blood drawn under a protocol approved by the Institutional Review Board. All patients satisfied the 1982 American Rheumatism Association revised criteria for the classification of SLE [15]. Sera were stored at −70°C.

Ro ELISA
A solid-phase immunoassay for anti-Ro was performed as previously described [9,16].

Western immunoblot
These assays were performed using a previously reported protocol that separates the 52-kD Ro protein from the 48-kD La protein [17]. Using an identical protocol, eluates from Ro-MAP columns were subjected to SDS–PAGE, transferred to nitrocellulose and probed with sera from rabbits immunized with 60-kD Ro in order to determine whether 60-kD Ro was present in these eluates.

Ro MAP
Multiple antigenic peptides (MAP; Applied Biosystems, Foster City, CA) were produced by standard methods in the University of Oklahoma Molecular Biology Core Facility, under the direction of K. Jackson. The sequences of these 21 constructs were based on the amino acid sequence of 60-kD Ro molecule [18,19] and the previously identified epitopes of 60-kD Ro [8,9,11,21]. The amino acid composition and sequence position within 60-kD Ro were as previously reported [11,22].

Ro-MAP ELISA
The Ro-MAP were used in a solid-phase assay, as described [11,22]. A panel of 20 normal persons and 20 SLE patients without anti-Ro, as assessed by the Ro ELISA, were used as controls. An optical density (OD) value was considered positive when greater than 3 s.d. above these controls and at least twice the average OD for each individual Ro-MAP.

Double immunodiffusion
These assays were performed in 1% agar with 3 μg of a Ro-MAP per well as antigen and 40 μl of sera per well. When 60-kD Ro was used as antigen, 10 μg were added per well as described [23,24].

Affinity chromatography
Selected Ro-MAP were linked to preactivated Sepharose 4B (Sigma Chemical Co., St Louis, MO) according to instructions provided by the manufacturer. Patient sera, diluted 1:10 in PBS, were passed over the column. The column was washed with at least 10 column-volumes of PBS. The unbound fraction was recovered, concentrated by centrifugation in a Centricon 10 (Amicon, Beverly, MA) and stored. Elution of bound antibody was accomplished with sodium thiocyanate pH 3·0. Fractions (0·5 cm^3) were collected and monitored for protein content by absorption of light at 280 nm.

Rheumatoid factor assay
Purified Fc portion of immunoglobulin (Sigma) was coated in the wells of microtitre plates in carbonate buffer pH 9·6 at a concentration of 10 μg/ml. After washing four times with a PBS–Tween 20 (0·05%) solution, wells were blocked with 3% milk in PBS. Concentrated eluate from the Ro-MAP columns was then added, along with sera containing IgG and IgM rheumatoid factor that served as positive controls. Alkaline phosphatase-conjugated anti-human antibody, either IgG or IgM, was next placed in the wells after washing as before. Finally, substrate was added and OD read at 405 nm (MicroELISA Reader; Dynatech, Alexandria, VA).

Surface plasmon resonance
The BIAcore instrument (Pharmacia, Uppsala, Sweden) was used to determine binding of one Ro-MAP to another by measuring surface plasmon resonance (SPR). SPR is an optical phenomenon arising in thin metal films under conditions of total internal reflection. One reactant is immobilized to a carboxy-methylated dextran matrix attached to the sensor surface, which is in contact with a microfluidic cartridge. The other surface of the sensor, coated with a thin gold layer, faces the optical system. The SPR detector responds to refractive index changes if the bound ligand interacts with soluble molecules. PBS/P20 pH 7·4 was used as the eluent (running buffer) at a flow rate of 5 μl/min during immobilization and all analyses with BIAcore. P20 (Tween, supplied by Pharmacia Biotech) was used at a concentration of 0·005% in the running buffer to reduce non-specific binding of protein to the sensor chip. The sensor surface was prepared as follows. After equilibration with PBS, it was then activated with a 35-μl injection of a 1:1 mixture of 0·1 M *N*-ethyl-*N*′-(dimethylamino-propyl) carbodiimide (EDC) and 0·1 M *N*-hydroxysuccinimide (NHS). Then 30 μl of a specific Ro-MAP at a concentration of 100 μg/ml in 10 mM sodium acetate pH 5·0 were injected. Remaining N-hydroxysuccinimide esters were deactivated with a 7-min pulse of 1 M ethanolamine pH 8·5. Typically about 3000–4000 response units (RU) of the MAP were immobilized. Ro MAP or control MAP (at 2 μg/ml) were used as the analyte over the Ro-MAP-482 ligand on the sensor chip and the bound fraction regenerated with 10–20 μl of 3 M NaSCN. A single ligand surface was used for several analyses. For kinetic experiments, different concentrations of the Ro-MAP-482 were employed in an effort to obtain a low baseline RU. By injecting 7 μl of the NHS-EDC mixture and 10 μl of the MAP (1 μg/ml), we were able to lower the RU of the MAP on the sensor chip to about 800. Increasing concentrations of different Ro-MAP were then passed over the sensor-bound Ro-MAP-482 for kinetic studies. Several control MAP were also immobilized in different flow cells using an identical protocol. These MAP were also used as control analytes, and included MAP constructed from the sequence of the 48-kD La (or SSB) autoantigen (amino acid residues 25–44) and from the Sm-associated autoantigen B/B′ (PPPGMRPP and PPPGIRPP). Software supplied by the manufacturer was used in calculations of affinity constants.

RESULTS

We determined the epitopes of 60-kD Ro bound by 67 patients with SLE who have anti-Ro. The specific peptides bound by 10 representative patients are shown in Fig. 1. As can be seen, some patients bound many of the 20 epitopes, while other patients bound few. The peptide containing the amino acid residues 401–411 was a negative control. In previous investigations this sequence was not bound by naturally arising human anti-Ro [8,11], sera of animals immunized with purified 60-kD Ro (unpublished observations), anti-Ro produced in animals immunized with a viral protein [24], or animals immunized with 60-kD Ro peptides [22]. None of the 67 patients studied bound this peptide significantly more avidly than controls. The epitopes bound for all 67 anti-Ro-positive patients are shown in Table 1. We determined other serologic manifestations in these patients, including anti-La (or SSB), anti-Sm, anti-RNP, anti-P, and anti-52-kD Ro. There is no statistical relationship among these other antibodies and any single peptide epitope of 60-kD Ro.

Unexpectedly, we found that patient sera and some Ro-MAP produced a precipitin line in double immunodiffusion. The interaction of a single patient serum and the 21 Ro-MAP is shown in Fig. 2. These double immunodiffusion experiments demonstrate

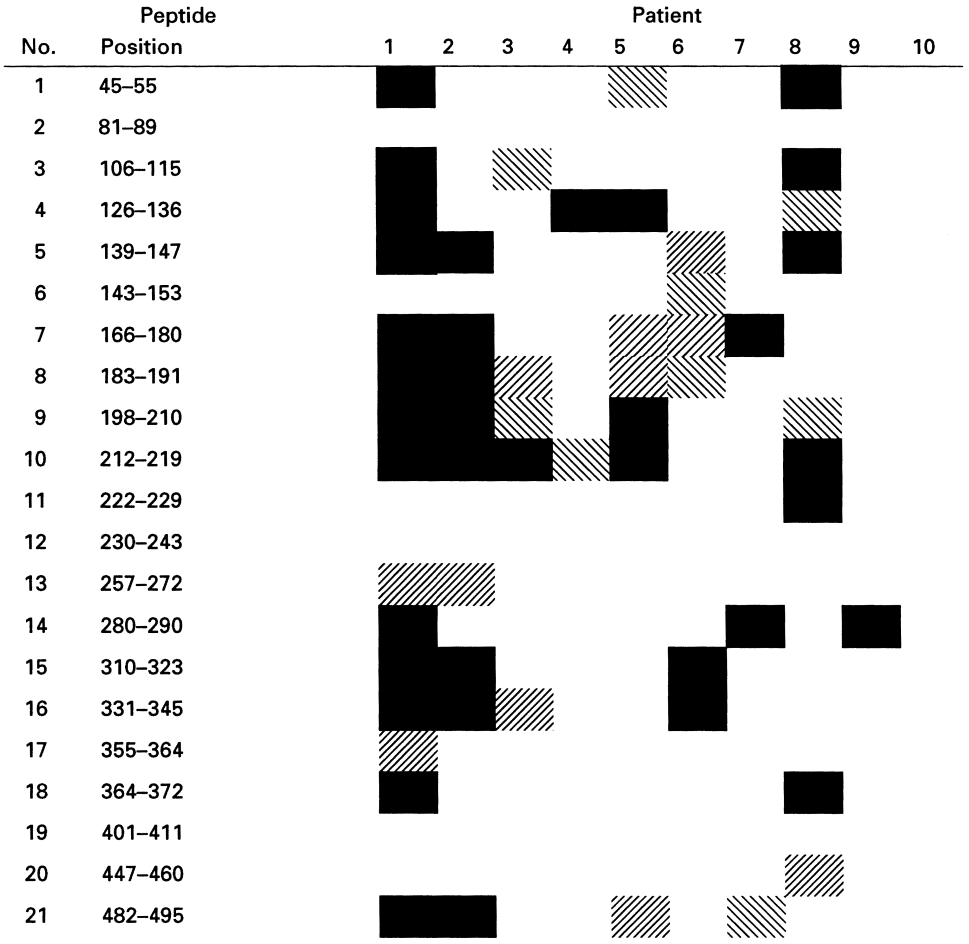

Fig. 1. Ro-multiple antigenic peptides (MAP) binding for 10 systemic lupus erythematosus (SLE) patients with anti-Ro. Binding is given as the number of s.d. above normal and anti-Ro-negative SLE patient controls on each of the 21 Ro-MAP epitopes based on criteria outlined in Patients and Methods. Patients are included with anti-Ro alone, anti-Ro plus anti-La, and anti-Ro plus anti-RNP, as well as patients with and without anti-Ro52. □, < 3; ▨, 3; ▧, 4; ■, 5.

that a line of identity or partial identity may be formed between adjacent Ro-MAP. No precipitin was formed when one of several MAP was used that had an unrelated (i.e. non-Ro-derived) peptide, nor was a line seen when the MAP backbone of poly-lysine was used in double immunodiffusion. Five peptides that represent 60-kD Ro epitopes are available that were not produced as MAP. No lines of identity formed when these monomeric peptides were used as antigen in double immunodiffusion (not shown).

The ability of sera to form a precipitin line with a given Ro-MAP was always associated with binding of that Ro-MAP in ELISA. As might be expected, the ELISA was a more sensitive measure of antibody than double immunodiffusion. Thus, some

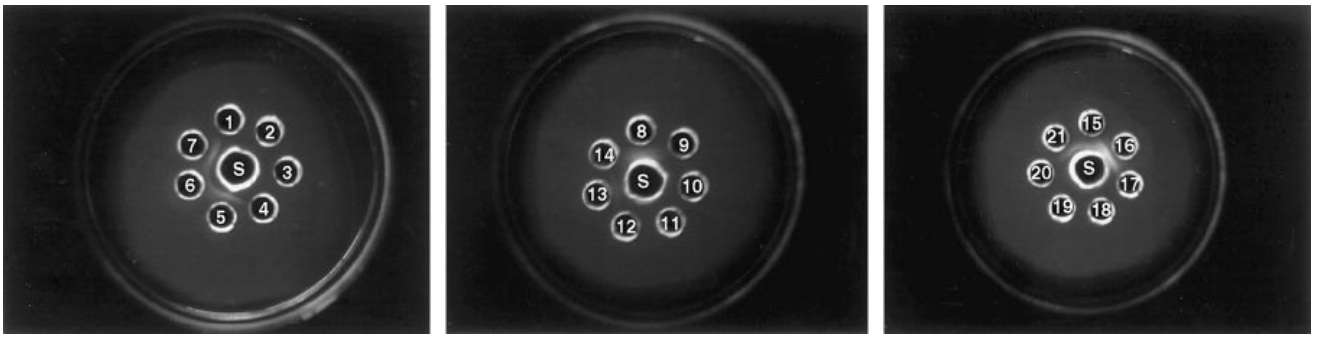

Fig. 2. Double immunodiffusion (Ouchterlony) of sera with the Ro-multiple antigenic peptides (MAP). An anti-Ro-positive patient serum is in the centre well with Ro-MAP in the surrounding wells of each gel. Each Ro-MAP is identified by number based the position within the Ro protein sequence.

Table 1. Ro-multiple antigenic peptide (MAP) ELISA results for 67 anti-Ro-positive systemic lupus erythematosus (SLE) patients divided according to other autoantibody specificities found in their sera

Peptide*	Anti-Ro alone, $n = 28$, no. positive	Anti-Ro/La, $n = 26$, no. positive	Anti-Ro/RNP, $n = 13$, no. positive
1/45/TYYIKEQKLGL	17	13	7
2/81/SQEGRTTKQ	5	7	3
3/106/STKQAAFKAV	9	12	5
4/126/TFIQFKKDLKES	14	13	7
5/139/MKCGMWGRA	8	10	5
6/143/MWGRALRKAIA	2	7	2
7/166/LAVTKYKQRNGWSHK	12	11	5
8/183/LRLSHLKPS	8	9	5
9/198/VTKYITKGWKEVH	12	10	4
10/212/LYKEKALS	16	9	5
11/222/TEKLLKYL	6	9	5
12/230/EAVEKVKRTKDELE	9	8	1
13/257/HLLTNHLKSKEVWKAL	8	9	2
14/280/ALLRNLGKMTA	9	12	6
15/310/NEKLLKKARIHPFH	6	7	3
16/331/YKTGHGLRGKLKWRP	9	10	4
17/355/AAFYKTFKTV	7	7	0
18/364/VEPTGKRFL	7	13	5
19/401/MVVTRTEKDSY	0	0	0
20/447/CSLPMIWAQKTNTP	5	6	0
21/482/ALREYRKKMDIPAK	14	12	5

*The peptides are numbered in consecutive order from the amino-terminus of the 60-kD Ro antigen. The position of the amino-terminal amino acid of each peptide and the sequence are given. For simplicity, in subsequent tables and figures peptides are named by their number only. The number of patients binding a particular peptide is shown. No single peptide was bound more frequently by one group compared with the other patients when tested with a χ^2 analysis and the Bonferoni correction for multiple comparisons.

Ro-MAP were bound in ELISA but not in double immunodiffusion. Conversely, no Ro-MAP formed a precipitin line that was not bound by the same serum in ELISA (Table 2).

Previously, we found that antibody binding short peptides from 60-kD is part of the response to the native molecule [11]. These data suggest that the overlapping peptide and the Ro-MAP techniques may identify antigenic peptides that are components of conformational epitopes of 60-kD Ro. The finding that anti-Ro sera formed lines of identity with Ro-MAP gave us a powerful method to test this hypothesis.

The problem was approached as follows. Patient sera were passed over Sepharose-Ro-MAP columns. Ro-MAP were selected that were bound by the patient sera that were passed over the columns. The column-bound and column-flow-through fractions were then tested for binding to the full panel of Ro-MAP peptides. In addition, each fraction was tested for anti-Ro activity.

Table 3 gives the results of the above experiments for four patients, each passed over three MAP-columns in separate experiments. Binding to the 21 60-kD Ro peptides is given for antibody eluted from each of the Ro-MAP columns. Of particular interest is that antibody eluted from a single Ro-MAP column binds not only to the peptide represented on the column, but also to other Ro-MAP. In fact, antibody from one patient eluted from a given Ro-MAP tends to bind the same set of peptides bound by the other patient serum eluted from the same peptide.

A quantitative assessment of the percentage of patient antibody bound to a given MAP-column indicated that a variable portion, from small to significant, of the total anti-Ro could be removed with passage of serum over a single Ro-MAP column. For selected peptides and patient sera, the percentage of anti-Ro removed ranged from 0·6% (patient 4 over 183–191 Ro-MAP) to 47% (patient 3 over 331–345 Ro-MAP). Remarkably, after absorption of antibody by passage over the 331–345 Ro-MAP column, 97% of total anti-Ro was removed from patient 2. When passed over a column linked with a Ro-MAP not bound by these sera, virtually no antibody was removed (see Table 3).

In order to determine whether the presence of rheumatoid factor might be responsible for the results obtained using the Ro-MAP column, we assayed eluates from the columns for rheumatoid factor. No rheumatoid factor was found in the eluates from Ro-MAP-331 column for the sera used in the above experiments (not shown). In addition, there was no 60-kD Ro antigen found in the eluates (not shown).

These data suggested that specific sets of Ro-MAP were bound by the same antibody. Because precipitin lines were formed between sera and Ro-MAP, we were in a position to demonstrate this was the case. Double immunodiffusion assays were performed using the data available from the affinity chromatography experiments. Groups of Ro-MAP for which antibody was adsorbed by a single Ro-MAP column were placed in adjacent wells. We found that sera formed lines of complete identity among such Ro-MAP. In contrast, when Ro-MAP were used whose antibody was not

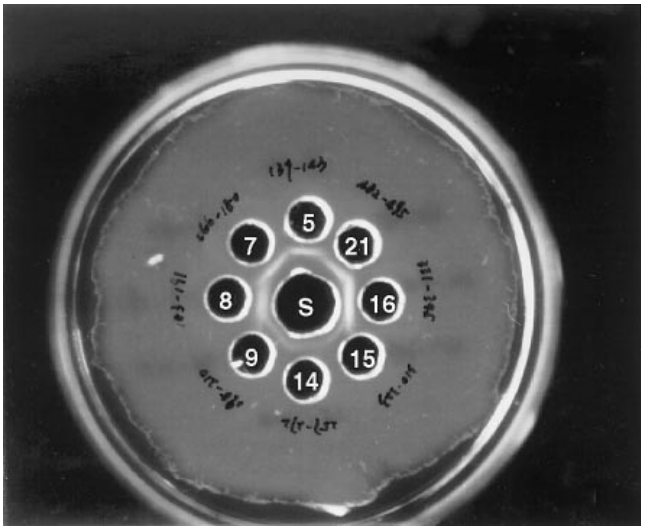

Fig. 3. Double immunodiffusion that demonstrates both lines of identity and lines of partial identity among the Ro-multiple antigenic peptides (MAP) and patient sera. When this serum is passed over a column with Ro-482 (no. 21) bound, the eluate binds Ro-MAP-139 (no. 5), -166 (no. 7), -183 (no. 8), -331 (no. 16) and -482 (no. 21). Meanwhile, when passed over a Ro-198 (no. 9) column, antibodies binding Ro-MAP-198 (no. 9), -280 (no. 14), and -310 (no. 15) can be eluted from the column. These two groups of Ro-MAP form lines of identity among the members of each group and lines of partial identity between the two groups.

Table 2. Comparison of binding of Ro-multiple antigenic peptides (MAP) in a solid phase assay with precipitin line formation in double immunodiffusion for four patient sera. The sequence position of each Ro-MAP is shown in Table 1

Peptide	Patient 1, ELISA/DIF	Patient 2, ELISA/DIF	Patient 3, ELISA/DIF	Patient 4, ELISA/DIF
1	+/–	+/–	–/–	–/–
2	–/–	–/–	–/–	–/–
3	–/–	+/–	–/–	–/–
4	–/–	–/–	–/–	–/–
5	+/+	+/+	+/+	+/+
6	+/+	+/+	+/+	+/+
7	+/+	+/+	+/+	+/+
8	+/+	+/+	+/+	+/+
9	+/–	+/+	+/+	+/+
10	+/–	+/–	–/–	–/–
11	–/–	–/–	–/–	–/–
12	–/–	–/–	–/–	–/–
13	+/+	+/+	–/–	–/–
14	+/–	+/–	–/–	–/–
15	+/+	+/+	+/–	+/+
16	+/+	+/+	+/+	+/+
17	–/–	–/–	–/–	–/–
18	–/–	+/–	–/–	–/–
19	–/–	–/–	–/–	–/–
20	+/–	–/–	–/–	+/–
21	+/+	+/+	+/–	+/–

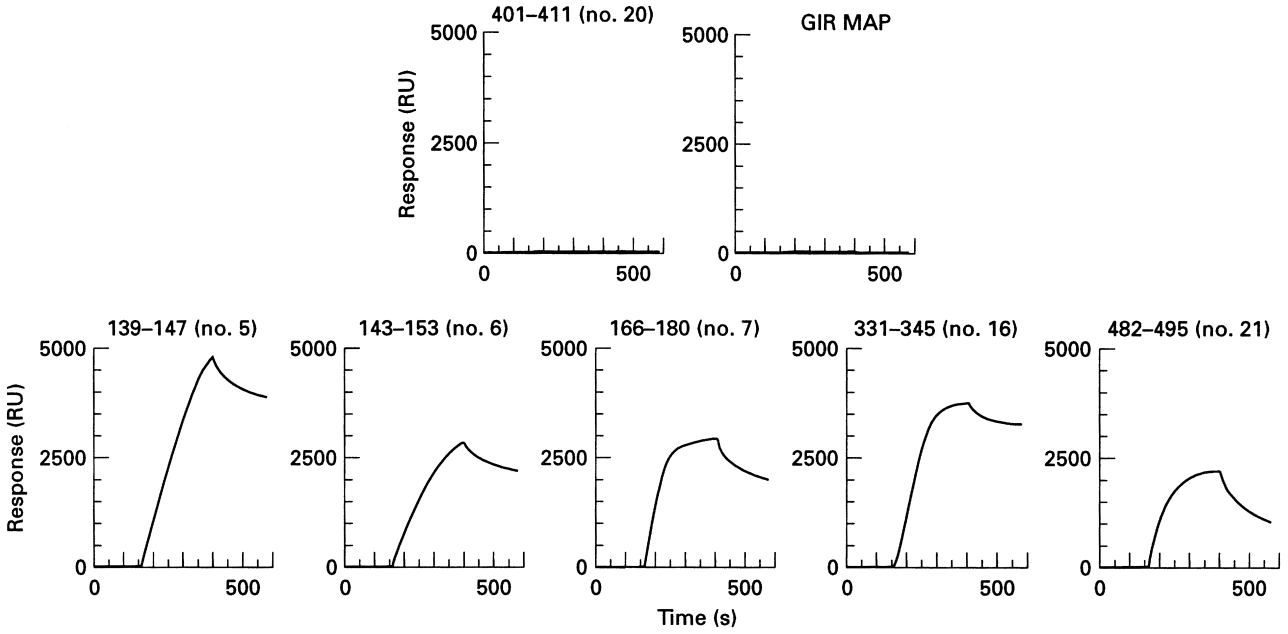

Fig. 4. Plasmon surface resonance binding of the Ro-multiple antigenic peptides (MAP) peptides to Ro-MAP-482. Ro-MAP-139 (no. 5), -143 (no. 6), -166 (no. 7), and -331 (no. 16) bound to Ro-MAP-482 in these assays, and their binding profile is shown. Other Ro-MAP did not bind Ro-MAP-482, except that Ro-MAP-482 bound itself (bottom right). MAP constructed from the sequence of the 48-kD La (or SSB) antigen (not shown), from the Sm antigen B/B′ (top right) and Ro-MAP-401 (top left) served as controls and did not bind Ro-MAP-482. RU, Response units.

Table 3. Passage of sera over affinity columns with Ro-multiple antigenic peptide (MAP) covalently linked results in absorption of antibody binding multiple Ro-MAP from each sera

	Peptide																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	%
Patient 1																						
MAP 8	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	44
MAP 16	−	−	−	−	+	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	20
MAP 19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
Sera	+	−	−	−	+	−	+	+	+	+	−	−	−	+	+	+	+	−	+	−	+	N/A
Patient 2																						
MAP 8	−	−	+	−	+	−	+	+	+	+	−	−	+	−	+	+	−	−	−	−	−	20
MAP 16	+	−	+	−	+	−	+	+	+	+	−	−	−	+	+	+	−	−	−	−	−	97
MAP 19	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	7
Sera	+	−	+	−	+	+	+	+	+	+	−	−	+	+	+	+	−	+	−	−	+	N/A
Patient 3																						
MAP 8	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	0.7
MAP 16	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	47
MAP 19	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0·08
Sera	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	N/A
Patient 4																						
MAP 8	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	0·6
MAP 16	−	−	+	−	+	−	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−	38
MAP 19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
Sera	−	−	−	−	+	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	N/A

Binding to each peptide by eluate from three different Ro-MAP columns is shown for each of four sera along with binding of the Ro-MAP for the sera themselves. The Ro-MAP-401 column was a negative control. This peptide is not bound by these, or any other anti-Ro, sera.

absorbed over this column, crossing lines or lines of partial identity were formed (Fig. 3). These data indicate that some Ro-MAP, to which antibody is absorbed out of the sera by a single Ro-MAP, are bound by the same anti-Ro antibodies.

We hypothesized that if certain sets of peptides are bound by a common component of anti-Ro and make up a putative tertiary epitope, then these peptides are in close proximity and might physically interact as part of the native structure of the protein. In order to test this hypothesis, we employed SPR.

Ro-MAP-139, -143, -166, -183, -198, -331 and -482 frequently formed precipitin lines of identity among themselves, and could mutually absorb the others of the group from sera (see Figs 2 and 3 and Table 3). We chose to study the binding of all the Ro-MAP to Ro-MAP-482. This peptide was bound to the surface of the plasmon resonance biosensor, and each of the Ro-MAP was tested individually for binding. As shown in Fig. 4, we found that Ro-MAP-139, -143, -166 and -331 had easily demonstrable binding to Ro-MAP-482. In addition, Ro-MAP-482 bound itself in this assay. Other Ro-MAP did not bind Ro-MAP-482, nor did control MAP from 48-kD La/SSB or from the Sm B/B′ antigen.

We estimated the binding affinity of Ro-MAP-139 and Ro-MAP-166 for Ro-MAP-482 by use of SPR. Ro-MAP-482 was bound to the sensor surface at the lowest concentration which produced a reliable signal in order to reduce mass transport effects. In addition, the flow rate of either Ro-MAP-139 or Ro-MAP-166 was varied from 5 μl/min to 20 μl/min. There was no apparent mass transport effect across these flow rates. For the Ro-MAP-482 and Ro-MAP-139 interaction an overall Keq of $1{\cdot}2\times10^6$ was obtained and for Ro-MAP-166 the value was $1{\cdot}3\times10^6$. The Kd and Ka values at each flow rate studied are shown in Table 4.

Table 4. Plasmon surface resonance kinetic values for the binding of Ro-MAP-482 (no. 21) with either Ro-MAP-143 (no. 6) or Ro-MAP-166 (no. 7)

	Flow rate (μl/min)		
	5	10	20
Ro-MAP-143 (no. 6)			
Kd ($\times10^{-3}$)	1·78	1·37	1·59
Ka ($\times10^3$)	5·08	4·79	8·73
Keq ($\times10^6$)	2·85	3·49	5·49
Ro-MAP-166 (no. 7)			
Kd ($\times10^{-3}$)	2·09	1·91	1·75
Ka ($\times10^3$)	3·58	3·62	7·99
Keq ($\times10^6$)	1·71	1·89	4·50

DISCUSSION

The epitopes of 60-kD Ro have been studied extensively by a number of methods. Several investigations have used recombinant gene clones [25,26] or protease digestion of purified protein [9,21] to produce peptide fragments. We used overlapping octapeptides [8,11,21] and others have used longer peptides that partially overlap [10]. Naturally, as these studies have used widely varying

techniques, the details of the results have differed. Nonetheless, in a qualitative sense there is agreement. Human anti-Ro autoantibodies bind the 60-kD Ro protein at many sites along the primary sequence in a complicated way.

Each of the methods used has shortcomings, but clearly they all suffer because much of the antigenicity of the molecule is lost with denaturing. Thus, the antibodies presumably interact more avidly with conformational epitopes on the surface of the native molecule. However, we find binding to overlapping octapeptides even by sera that do not bind denatured whole 60-kD Ro in immunoblot [8,11,21]. In addition, in apparent contradiction, we found that purification of antibody with native antigen results in enrichment for antibody binding overlapping octapeptides [11].

These data led to the present study. We found that non-adjacent segments of the primary sequence were bound by the same subset of anti-Ro. That this was the case was shown by affinity chromatography and by double immunodiffusion. The primary sequences bound by the same antibody have no obvious amino acid sequence identity or repeating motifs.

There are several possible interpretations. For example, the peptides bound by the same antibodies could assume an identical, or near identical, conformation, and thus be bound by the same antibody [27,28]. On the other hand, that there would be multiple peptides assuming the same conformation and that there would be multiple sets of such peptides in the 60-kD Ro sequence is implausible. The presence of rheumatoid factor in the sera might explain some of the findings after passage of sera over Ro-MAP columns. However, one would expect the entire spectrum of anti-Ro, binding all the identified peptide epitopes bound by the sera, if rheumatoid factor was mediating binding of multiple specificities to the columns. In fact, we found no rheumatoid factor or 60-kD Ro antigen in the eluates. Thus, neither rheumatoid factor nor immune complexes were mediating the findings reported here.

Another explanation is that peptides identified together on the basis of the affinity chromatography and immunodiffusion experiments are component parts of tertiary epitopes. Such peptides are then hypothesized to be in close proximity to each other on the surface of the Ro molecule. This structure found in the native molecule is bound most avidly by antibody. However, when produced as short peptides and used in immunoassay, these peptides can assume many conformations. Among these should be the one present in the native molecule. When this particular conformation is assumed, then the conformationally dependent anti-Ro antibody can bind the peptide. Longer peptide fragments, such as we [9] and others [10,25,26] have used, are likely to be more constrained based on energy considerations, so that the native conformation of a given short region may rarely, if ever, be obtained.

Other data support the notion that these groups of peptides are the components of tertiary epitopes. We find that at least some of the peptides that are identified by immunodiffusion and affinity chromatography bind each other. This suggests that such peptides are adjacent in the native structure of 60-kD Ro and that their interactions contribute to the binding energy that constitutes the tertiary structure. Some peptides, such as Ro-MAP-198, were associated with Ro-MAP 482 in other assays but did not bind Ro-MAP-482. This may be the result of each of these peptides contributing to the binding energy of a tertiary epitope, but not all directly interacting with each of the others. An exhaustive analysis of the intramolecular protein–protein interactions of 60-kD Ro via these techniques may enhance knowledge of structure, function and immunogenicity of the molecule.

A significant portion of anti-Ro activity is absorbed from some sera when passed over a single Ro-MAP affinity column. These data further suggest that the overlapping octapeptide and Ro-MAP techniques identify the majority of anti-Ro antibody in sera of patients. There is no evidence that Ro-MAP peptides are non-specifically binding antibody. Sequences not bound by the patients remove little to no anti-Ro from the sera. In addition, when sera with other specificity, such as anti-Sm, are passed over the Ro-MAP column, no antibody is bound and removed.

In conclusion, epitopes of 60-kD Ro can be identified in a number of ways. The approach we have taken allows determination that certain non-adjacent regions of the primary sequence that have no shared sequence are bound by the same anti-Ro autoantibody. In some cases, these sequences also interact with each other, providing further evidence of their close relationship. Such knowledge explains the discrepancy that the denatured molecule is bound poorly but that short peptides from the same molecule are bound well. Thus, at least for anti-Ro and the techniques used, the distinction between primary and conformational antigenic determinants is largely semantic.

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