Routsias-JG; Tzioufas-AG; Sakarellos-Daitsiotis-M; Sakarellos-C; Moutsopoulos-HM ***Η χαρτογράφηση επιτόπων του αυτοαντιγόνου Ro/SSA 60kD αποκαλύπτει νοσοειδικά μοτίβα δέσμευσης αντισωμάτων.*** Eur-J-Clin-Invest. 1996 Mar; 26(6): 514-521

Τα αυτοαντισώματα κατά της Ro/SSA 60kD πρωτεΐνης ανευρίσκονται συχνά στον ορό ασθενών με πρωτοπαθές σύνδρομο Sjogren (π. σ. Sj) και συστηματικό ερυθηματώδη λύκο (ΣΕΛ). Ο σκοπός της μελέτης αυτής ήταν η ταυτοποίηση των κυρίων γραμμικών αντιγονικών επιτόπων της πρωτεΐνης. Για το λόγο αυτό συντέθηκαν αλληλοεπικαλυπτόμενα 22-μερή πεπτίδια που κάλυπταν όλο το μήκος του μορίου (538 αμινοξέα), με αλληλοεπικάλυψη 8 αμινοξέων. Η σύνθεση έγινε σε ράβδους πολυαιθυλενίου σύμφωνα με την τεχνική του Geysen. Μελετήθηκαν 3 ομάδες ορών. Η πρώτη ομάδα αποτελείται από 5 ορούς ισάριθμων ασθενών με (4 με ΣΕΛ και 1 με π. σ. Sj) που περιείχαν μόνο αντισώματα κατά Ro60kD. Η δεύτερη ομάδα περιελάμβανε 4 ορούς ισάριθμων ασθενών με π. σ. Sj που είχαν δραστικότητα κατά Ro60kD και La/SSB. Η τρίτη ομάδα (ελέγχου) περιελάμβανε 3 φυσιολογικούς ορούς και έναν ορό ασθενούς με π. σ. Sj και αντισώματα κατά Ro52kD. Βρέθηκε ότι οροί ασθενών με ΣΕΛ αντιδρούν με το πεπτίδιο TKYKQRNGWSH-KDLLRSHLKP (169-190) και οι οροί ασθενών με π. σ. Sj με το πεπτίδιο ELYKEKALSVETEKLLKYLEAV (211-232) της αλληλουχίας του Ro60kD αντιγόνου. Περιορισμός των επιτόπων στο ελάχιστο απαραίτητο μήκος αναγνωρίσεως από τα αυτοαντισώματα, έδειξε ότι οι ακριβείς αντιγονικές περιοχές είναι οι NGWSHKDLLR (175-184) και KALSVETEKLLKYLEAV (216-232) αντίστοιχα. Πειράματα αναστολής με φυσικό Ro60kD αντιγόνο και διαλυτά πεπτίδια p175-184 και p216-232 επιβεβαίωσαν την ειδική πρόσδεση αυτοαντισωμάτων στις περιοχές αυτές. Συγκριτική μελέτη της αλληλουχίας των επιτόπων, χρησιμοποιώντας βάσεις δεδομένων αλληλουχίας πρωτεϊνών έδειξε ότι ο επίτοπος NGWSHKDLLR (175-184) προσομοιάζει με την περιοχή YWNSQKDLLQ της β-αλυσίδας των αντιγόνων HLA-DR.

Συμπερασματικά, το αντιγόνο Ro60kD, περιέχει 2 διακριτούς αντιγονικούς επιτόπους, οι οποίοι είναι ειδικοί ο ένας για τον ΣΕΛ και ο δευτερός για το π.σ. Sj. Ο επίτοπος175-184 προσομοιάζει με πεπτίδιο της β αλυσίδας των HLA-DR αντιγόνων και η βιολογική σημασία της ομοιότητας βρίσκεται ακόμα υπό διερεύνηση.

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Epitope mapping of the Ro/SSA60KD autoantigen reveals disease-specific antibody-binding profiles

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Abstract. Anti-Ro60KD autoantibodies are commonly found in sera from patients with primary Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE). In order to identify the epitopes of this autoantigen, 22-mer, synthetic peptides overlapping by eight residues, and covering the entire sequence of the Ro60KD autoantigen were prepared. Three groups of sera were evaluated according to their autoantibody specificities. The first group consisted of monospecific anti Ro60KD sera from four patients with SLE and one with SS, the second one was composed of anti-Ro60KD + anti-La(SSB)-positive sera from four patients with SS and the third group included three normal sera and one anti Ro52KD serum. It was found that sera from SLE patients interact with a common antigenic site spanning the sequence TKYKQRNGWSHKDLLRSHLKP (169–190) of the Ro60KD protein. On the other hand, sera from SS patients recognise the ELYKEKALSVETEKLLKYLEAV (211–232) region of this autoantigen. Determination of the minimal required peptide length for optimal antibody recognition showed that the defined epitopes can be shortened to the NGWSHKDLLR (175–184) and KALSVETEKLLKYLEAV (216–232) sequences respectively. Inhibition experiments using the Ro60KD antigen and soluble peptides corresponding to the 175–184 and 216–232 segments further confirmed the specific antibody binding. These results, although only a small number of sera were used, indicate that the Ro60KD autoantigen, which is not characterized by disease specificity, contains two discrete epitopes specifically recognized from SLE and SS patient sera. Finally, the sequence similarity of the NGWSHKDLLR (175–184) epitope with some of the HLA haplotypes, associated with anti-Ro response, deserves to be noted.

Keywords. AntiRo/SSA, epitope mapping, molecular mimicry, Sjögren's syndrome, systemic lupus erythematosus.

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Introduction

Ro ribonucleoproteins (RoRNPs) are particles composed of several proteins complexed with RNA polymerase III transcribed cytoplasmic RNAs (YRNAs) [1]. They are present in a variety of vertebrate species and cell types [2] but their biological function(s) remain unknown. These particles were discovered as common targets in certain autoimmune rheumatic diseases, such as Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) [3]. Although it is not yet known what leads to the breakdown of immunological tolerance and formation of antibodies against these self antigens, existing evidence suggests that anti-Ro autoantibodies have immunopathogenic potential [4]. The presence of anti-Ro antibodies is also associated with certain HLA haplotypes, especially with HLA-DR3, -DRw53, and DQ1/DQ2 heterozygosity [5, 6].

Humoral autoimmune response is primarily directed against the protein component of the RoRNP, which is composed of at least three immunologically distinct proteins, namely the Ro/SSA60KD, the Ro/SSA52KD and the La/SSB protein [1]. Antibodies against the Ro60KD protein are frequently found in both SLE and primary SS patients' sera, whereas antibodies to Ro52KD and La/SSB are detected more frequently in SS sera [7].

Several techniques have been used to map B-cell epitopes in Ro60KD protein. They include testing of autoimmune sera against overlapping synthetic peptides by enzyme linked immunosorbent assay (ELISA) [8] or with deletion mutants of recombinant proteins by Western blot or by immunoprecipitation [9]. These studies have yielded conflicting results so far, which can be attributed to several reasons, such as the type of assay used, the selection of patients' sera and the presentation of antigenic fragments [10]. Taking into account that a considerable amount of anti-Ro60KD antibodies are targeting conformational determinants [11], it appears that our knowledge of B-cell epitopes of the Ro60KD molecule remains rather limited. Nevertheless, owing to the clinical significance and the potential pathogenic role of the anti Ro/SSA autoantibodies, identification of the major antigenic determinants of

Ro60KD can provide important insights into our understanding of autoimmunity and could ultimately prove useful for the development of diagnostic tests.

In the present report, the linear antigenic determinants of the Ro60KD molecule have been investigated using large (22-mer) overlapping synthetic peptides, according to the multipin epitope mapping strategy based on two previously published sequences [12, 13]. It is shown that antibodies to Ro60KD of patients with SLE and primary SS preferentially recognized distinctly different epitopes on the molecule. The minimal required antibody-binding sequences of these epitopes and sequence similarities with other molecules are also reported.

Materials and methods

Sera and IgG purification.

A panel of sera from patients with SLE and primary SS was tested for their autoantibody specificity with counter immunoelectrophoresis, immunoblot and ELISA. All patients with SLE fulfilled the diagnostic criteria of the American College of Rheumatology [14] and those with primary SS the preliminary criteria of the European Union study group [15]. Sera that gave strong anti-Ro reaction by all methods and a clear band pattern in immunoblot were selected and divided into two groups. The first group consisted of monospecific anti Ro60KD sera from four patients with SLE and one with primary SS. The second group was composed of anti-Ro60KD and anti-La-positive sera from four patients with primary SS. The control group included three normal sera and one anti-Ro52KD serum from a patient with primary SS. IgG from all sera, purified by protein A–Sepharose column, was concentrated and dialysed against phosphate-buffered saline (PBS), pH 7·3. Concentrations of IgG were evaluated by nephelometry (Beckman Instruments, Brea, CA, USA).

Peptide synthesis

(a) A total of 119 synthetic peptides were prepared in duplicate by the multipin peptide synthesis of Geysen *et al.* [16]. The synthesis was performed on prederivatized polyethylene pins (Cambridge Research Biochemicals, UK) and the protocol was based on the principles of the solid-phase peptide synthesis of Merrifield [17], using the $N\alpha$-fluorenylmethoxycarbonyl (FMOC) protecting group strategy. For the accurate dispensing of different solutions at the coupling stage, self-made computer software was used. In each synthesis cycle control peptides $PLAG(G)_n$ and $GLAG(G)_n$ of various lengths ($n = 0$, 6, 12 and 18) were synthesized. All $PLAG(G)_n$ control peptides were specifically recognized by anti-PLAG monoclonal antibodies, (Cambridge Research Biochemicals) while GLAG(G)*n* control peptides did not react.

(b) Solid-phase synthesis of the NGWSHKDLLR (175–184) and KALSVETEKLLKYLEAV (216–232) peptides, using benzhydrylamine anchor-bond [18] (resin substitution [19] estimated 0·42 and 0·46 mq/g^{-1} respectively) and N^{α}-Boc/benzyl side-chain protection, was carried out by standard methods. Amino acid couplings were performed by the dicyclohexylcarbodiimide (DCC)–Hydroxybenzotriazole (HOBt) procedure using a ratio of amino acid/DCC/HOBt/resin(3 : 3 : 3 : 1). Deprotection of the N^{α}t-Boc protecting group was performed using trifluoroacetic acid followed by diisopropylethylamine for neutralization. After synthesis, the peptides were cleaved from the resin with anhydrous hydrogen fluoride in the presence of anisole and phenol (10% v/v) as scavengers at 0°C for 1 h. The peptides were extracted from the resin using 2 M acetic acid and after lyophilization were subjected to chromatographic purification using Sephadex G-25 equilibrated with 2 M aqueous acetic acid. Elution of the peptides was performed using a homogeneous mixture on *n*-butanol–pyridine–acetic acid–H_20(9:3:2:4:,v/v)(BPyAW). Best purity was achieved when the eluent volume, passed through the column before loading the peptides, corresponded to the half-bed column. The peptides were homogeneous as determined by thin-layer chromatography in BPyAW (9:3:2:4) [Rf(175–184) = 0·27, Rf(216–232) = 0·35 and BPyAW (5:5:1:4) (Rf (175–184) = 0·47, Rf(216–232) = 0·54]. The overall yield after purification ranged from 40% to 50%. Appropriate one-dimensional and two-dimensional ^{1}H-nuclear magnetic resonance (NMR) spectra confirmed the identity and the purity of the peptides.

Cell extract and immunoblotting

Cytoplasmic extract was prepared from cultured HeLa cells as described by Habets *et al.* [20]. Samples of extracts were applied to polyacrylamide gel sodium dodecyl sulphate (SDS) electrophoresis, followed by electrotransfer to nitrocellulose. The nitrocellulose blots were stained with Ponceau S and cut into strips, which were blocked with non-fat milk5% at 4°C overnight. After washing, anti-human IgG conjugated to horseradish peroxidase was added (1:1000 dilution) in blocked buffer and allowed to react for 1 h. The colour was developed by adding a substrate solution of 4-chloro-1-naphthol to the strips.

Elisa

(a) The levels of anti-Ro/SSA antibodies in patient sera were determined by commercially available ELISA (Diastat anti-Ro, Shield Diagnostics, London UK) using microtitre wells coated with affinity-purified bovine Ro/SSA antigen, according to the manufacturer's instructions.

(b) Peptides covalently attached to polyethylene rods were tested for antibody binding by ELISA in 96-well microtitre plates. Rods were immersed in 10 nM sodium phosphate buffer, pH 7·2, 150 mM sodium chloride (PBS) containing 0·1% Tween 20, 1% albumin and 1% ovalbumin to block non-specific binding. The IgG, diluted with the above buffer (200 μg mL^{-1}), was added

to the wells and incubated overnight at 4°C. After washing with PBS containing 0·5% Tween 20,anti-human IgG, conjugated to horseradish peroxide (1:1000 dilution) in blocking buffer, was added and incubated for another 1 h at 20°C. The rods were again washed and the presence of antibodies was detected using a substrate solution of 2,2′-azino-*cis*-3-ethylbenzothiazoline sulphonic acid (ABTS) and the absorbance of the produced colour was measured at 405 nm. Subsequently, the bound antibodies were removed from the rods by sonication for 30 min in water bath with 0·1 M sodium dihydrogen phosphate, 1% SDS and 0·1% 2-mercaptoethanol at 60°C and the rods were used again or dried for storage.

Purification of the Ro/SSA antigen and inhibition assays.

Anti-Ro60KD affinity column was made by coupling purified IgG, specific for the NGWSHKDLLR peptide, to cyanogen bromide (CNBr)-activated Sepharose 4B using conventional methods. Human spleen extract was prepared as described previously [21] and applied to the anti-Ro60KD affinity column. Ro antigen was eluted with HCl–Glycine, pH 2·7. Its purity was evaluated by SDS gel electrophoresis and immunoblot. IgG samples (200 $mg\,mL^{-1}$) were incubated with the soluble peptide (100 $\mu g\,mL^{-1}$) according to Merrifield's method, or purified human Ro60KD antigen (10 $\mu g\,mL^{-1}$) for 1 h at room temperature, before application of ELISA tests.

Computer predictions and homology search

The flexibility, hydrophilicity and B-cell antigenic profiles using Ro60KD primary structure, were made by the EPIPLOT program. Prediction of the secondary structure of the Ro60KD was produced using the PHDsec profile neural network method [22]. The antigenic peptide sequences were compared against the SWISS-PROT, NBRF/PIR,PRF and GENPEPT databases using Fasta [23] and Smith–Waterman [24] algorithms at GENOMENET-FASTA and EMBL-FASTA, -BLITZ servers. The non-identical amino acids were scored with PAM 250 and PAM 100 matrices [25] and the gap inclusions were allowed in Smith-Watermans searching. HLA class II sequences were obtained by Marsh and Bodmer's compilation in the EMBL database.

RESULTS

Identification of the antigenic sites

Thirty-eight 22-mer, peptides, covering the entire sequences of the Ro60KD protein, as determined by Duetscher *et al*., were synthesized in duplicate. An additional 22 mer peptide, representing the main difference between Deutscher's and Ben-Chetrit's reported

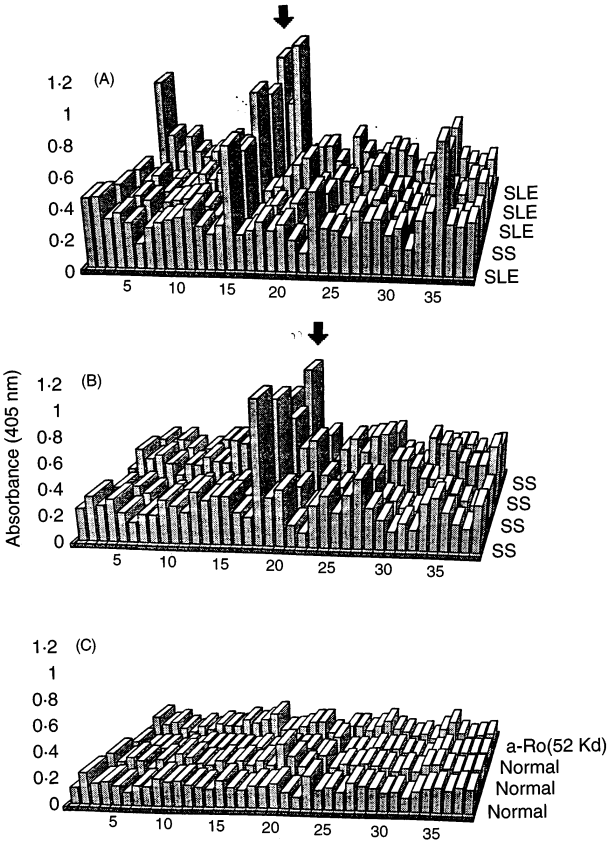

Figure 1 Epitope mapping of the Ro60KD protein. (A). Binding of the 22-mers to anti-Ro60KD-positive sera (systemic lupus erythematosus; SLE). The arrow indicates the peptide TKYKQRNGW-SHKDLLRLSHLKP (169–190 in Ro60 sequence). (B) Binding of the 22-mers to anti-Ro60KD + anti-la(SSB) positive sera (primary Sjögren's syndrome; SS). The arrow denotes the peptide ELYKE-KALSVETEKLLKYLEAV (211–232 in Ro60 sequence). (C) Binding of the 22-mers to normal sera and to one anti-Ro 52KD-positive serum. Columns represent the ELISA OPTICAL DENSITY at 405 nm of each 22 mer synthesized and rows correspond to individual sera tested.

sequences, was also synthesised. The antibody-binding pattern for all the peptides tested by ELISA against the three groups of sera are shown in Fig. 1. The sequence TKYKQRNGWSHKDLLRL- SHLKP corresponding to the 169–190 region of Ro60KD exhibited significant reactivity with all the anti-Ro monospecific sera (group 1) tested (5/5). One peptide sharing the 449–470 sequence of the antigen was recognised by 2/5 of the sera, whereas four more peptides gave positive reaction with 1/5 of the sera tested (Fig. 1A). Anti-Ro and anti-La-positive sera (group 2) gave a distinct epitope-scanning profile and the ELYKEKALSVETEKLLKYLEAV (211–232) peptide was clearly defined as the immunodominant region for 3/4 of the sera (Fig. 1B). Peptide 183–204 was positive for 1/4 of the sera and only one serum of this category was found inactive against all the synthesized 22-mers, due to the high background level. Normal sera as well as anti-Ro52KD serum (group 3) gave low absorbance values for all the peptides tested. (Fig. 1C).

Determination of the minimum required length of the antigenic determinants.

Refinement of the defined epitopes was performed by the synthesis of two sets of multiple-length peptides for each 22-mer antigenic site. The first set consisted of 18 peptides with decreasing length (from 22 to 5 amino acids). Each peptide was obtained by subtracting one amino acid, each time, from the amino termi-nus of the antigenic domain. Likewise, the second set of peptides used for the determination of the carboxyl end of the epitope included 18 peptides of multiple lengths, differing by one amino acid from the C-terminal part (Fig 2). Thus, restriction of the TKYKQRNGWSHKDLLRLSHLKP (169–190) antigenic determinant to the NGWSHKDLLR(175–184), by substraction of six amino acids from both ends of the 169-190 segment did not affect the antibody binding (Fig. 2A). Similarly, restriction of the ELYKEKALSVETEKLLK-YLEAV (211–232) antigenic site to the KALSVE-TEKLLKYLEAV (216–232) by deletion of five amino acids from the N-terminal part of the 211–232 segment did not cause any significant loss of antigenicity (Fig. 2B). Taking in to account that each individual serum gave a slightly different reaction pattern, the length of the minimum antibody-binding segments could be futher decreased by one or two amino acids depending on the serum used.

Inhibition of the antibody binding to the antigenic peptides.

Preincubation of serum, specific for the antigenic peptide ELYKEKALSVETEKLLKYLEAV (211–232), with the Ro60KD antigen, the KALSVETEK-LLKYLEAV (216–232) and the NGWSHKDLLR (175–184) soluble peptides led to 62%, 56% and 13% inhibition respectively. Antibodies against the antigenic peptide TKYKQRNGWSH-KDLLRLSHLKP (169–190) were inhibited at 54% by the Ro60KD antigen, at 24% by the NGWSHKDLLR and 8% by the KALSVETEKLLKYLEAV (216–232) peptide (Fig. 3)

Predicted features of the anti-Ro60KD immunodominant regions.

The two major anti-Ro60KD epitopes are located in the predicted α-helix central part of the molecule, between the RNA-binding motif RNP80 and a potential zinc-finger DNA-binding pattern. Cysteine residues capable of forming disulphide bonds were not found in the immunodominant regions of the Ro60KD protein. Both NGWSHKDLLR (175–184) and KALSVE-TEKLLKYLEAV (216–232) epitopes are hydrophilic sites [26] of medium flexibility [27] and possess a high ratio of amino acids commonly found in B-cell antigenic determinants [28].

Sequence similarities of the immunodominant epitopes.

The identification of the primary sequence of Ro60KD epitopes enabled the use of protein databases for searching similar sequences shared with other proteins of unrelated origin. Sequences with the highest similarity scoring drawn from these searches are summarized in

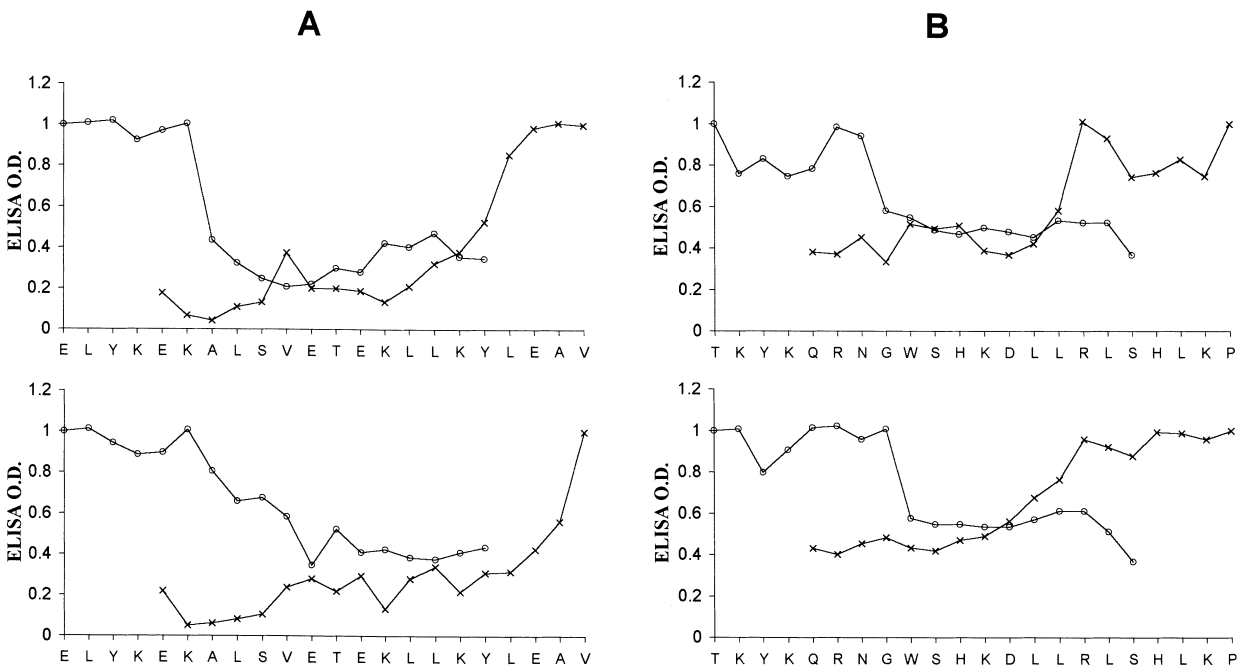

Figure 2. Restiction of the epitope length of the Ro60KD protein. (A) Minimal required amino acid sequence for binding to the anti-Ro60KD positive sera (systemic lupus erythematosus; SLE sera). (B) Minimal required amino acid sequence for binding to the anti-Ro60KD + anti-La solidus (SSB)-positive sera (primary Sjögren's syndrome; SS sera). The line starting from the left corresponds to the animo terminus and the line starting from the right corresponds to the carboxy terminus of the restricted epitopes.

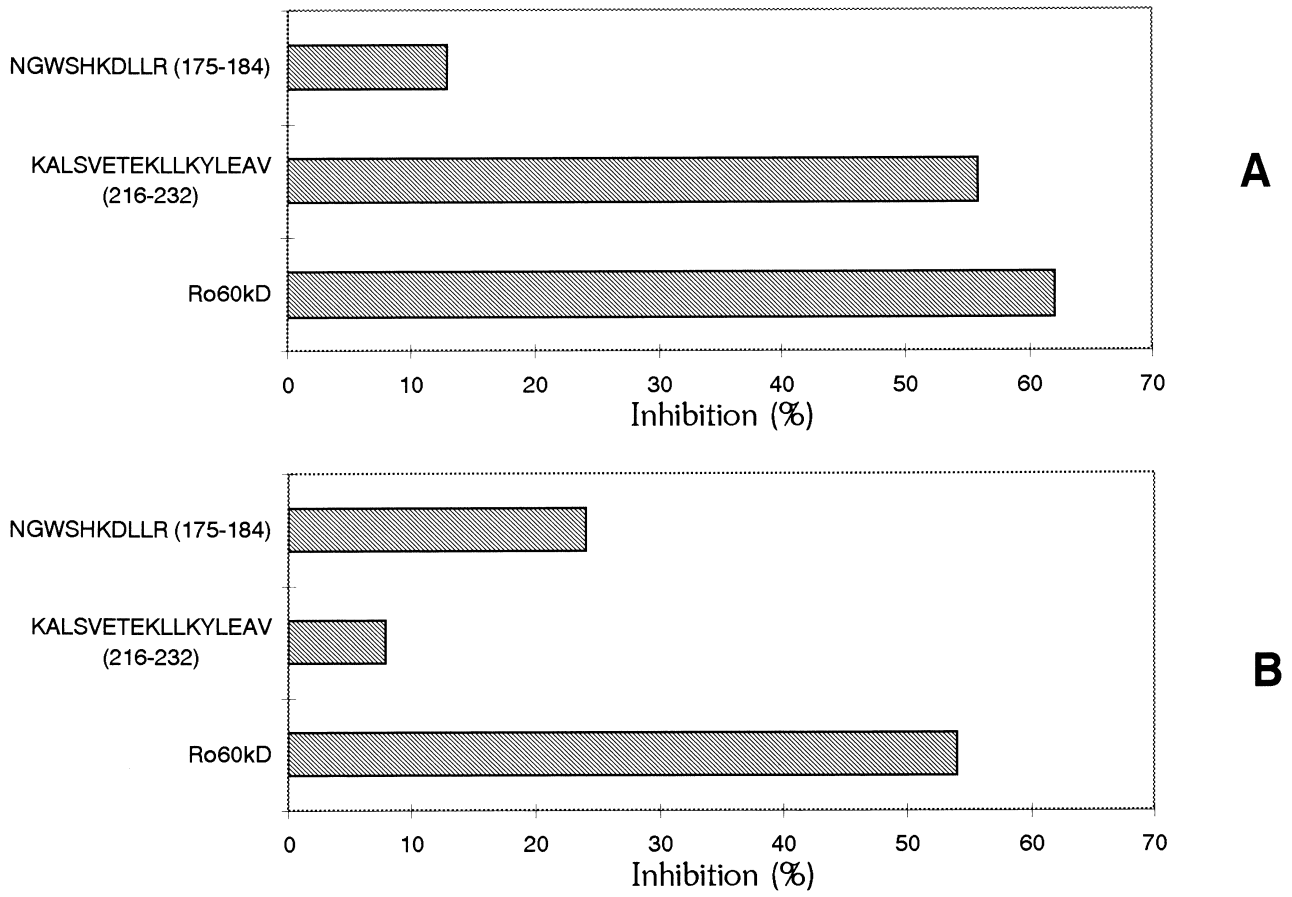

Figure 3. Inhibition studies of the peptides are shown. A, inhibition of the ELYKEKALSVETEKLLKYLEAV (the initially reactive 22-mer, SS-related peptide). ELISA, using as inhibitors the peptides NGWSHKDLLR (SLE-related epitope), KALSVETEKLLKYLEAV (SS-related epitope) and Ro60, are depicted. B, the inhibition rates of the TKYKQRNGWSHKDLLRLSHKP (the initially reactive 22-mer SLE-related peptide) ELISA using as inhibitors the peptides NGWSHKDLLR (SLE-related epitope), KALSVETEKLLKYLEAV (SS-related epitope) and Ro60 are shown.

Table 1. Amino acid sequences of the NGWSHKDLLR and KALSVE- TEKLLKYLEAV epitopes were found to be conserved in the xenopus Ro/SSA protein (100% and 84% sequence similarity respectively). The NGWSHKDLLR also had local identity with the adenovirus E4TF1 transcription factor (homologous also with the herpes simplex virus I GA-binding protein and the HLA class II β-chain). The latter presented a six amino acid identity, including a gap. Some of the alleles of the anti Ro/SSA-associated haplotypes posses sequences with local identity to the NGWSHKDLLR epitope.

The KALSVETEKLLKYLEAV epitope shared partial sequence similarity with the ribonucletide reductase of the African swine fever virus (relative to the pox viruses family) and the hypothetical 7·17 protein of *Salmonella choleraesuis*.

Discussion

The identification of B-cell epitopes recognized by autoimmune sera may offer important biological and practical applications. In fact, it can provide useful information on both the mechanism involved in the stimulation of autoantibody production and the nature of the structures that could be targeted by autoantibodies. In addition, identification of the sequence of a major epitope may considerably facilitate the detection of autoantibody reactivity, as synthetic antigenic peptides can be used as reliable substrates for autoantibody detection tests [29]. The application of these tests can possibly be useful to discriminate between subsets of a disease thus having a potential prognostic value.

In the present study the fine specificity of anti-Ro60KD antibodies derived from two different disease entities is presented. Using a panel of monospecific anti-Ro60KD, anti-Ro60KD and anti-La/SSB sera, two discrete antigenic profiles were defined. The sequence 175–184 is preferentially recognized by SLE sera. whereas the sequence 216–232 is the main target of primary SS sera. Although the number of sera used for the epitope mapping is rather small, owing to the restricted capability of pin-bound peptides in ELISAs with a large number of samples, it seems that this antibody recognition pattern indicates a disease specificity of the identified epitopes. The slightly different

Table 1. Sequence similarities between the NGWSHKDLLR (A) and the KALSVETEKLLKYLAEV (B) epitopes of the Ro60KD antigen and other proteins

Amino Acid sequence		Protein
NGWSHKDLLR		Ro/SSA protein, human
NGWSHKDLLR	*(A)*	Ro/SSA protein, *Xenopus laevis*
ILWSHLELLR		Adenovirus transcription factor, Human
YWNSQKDLLQ*		HLA class II (B-chain), human
NWRSDKDLLE*		Antigen E *Ambrosia Artemisiifolia*
KALSVETEKLLKYLEAV		Ro/SSA protein human
KELSPETEKVLKYLEAT		Ro/SSA *Xenopus laevis*
IIYSIEQERLLKYEKEV	(B)	Hyp. protein 7·17 *Salmonella chloeraesuis*
GALEVKRKELLKYLTAA		Zinc finger protein Human
KALNVDLNKLLQALNHH		Ribonucleotide reductase, African swine fever virus

* Gaps included.

epitope terminal ends for some sera of the same group can be attributed to fine specificity variations between human autoantibodies resulting from a different antibody repertoire or idiotypic variation.

Previous reports have shown that antigenically distinct molecular forms of the RoRNP complex, exist in a variety of cell types and species [21]. Antibodies against the denatured Ro/SSA are commonly found in SLE but are absent in SS sera [30]. Futhermore, anti-Ro/SSA antibodies in SLE (where anti-La/SSB) antibodies are not usually observed) are directed to a region, near the RNP-80 motif, which possesses structural characteristics essential for the formation of the Ro/La-RNP particle [31]. It appears, therefore, that humoral response in different autoimmune diseases might target different molecular forms of the Ro/SSA antigen. These observations give further support to our findings concerning the disease-specific antibody binding profile of the Ro60KD, epitopes.

The length of the reported minimum antigenic determinations (10 and 17 amino acid sequences respectively) is much higher than usually observed for linear epitopes. For instance, Geysen *et al.* claimed that 90% of the continued epitopes are of six amino acid length or even less [32]. One possible explanation for this is that the ends of an antigenically active peptide may represent parts of a small discontinuous (conformational) epitope. Alternatively, a certain peptide length or specific residues at the end of the peptide may be essential for the stabilisation of the epitope active conformation. It is also known that only long peptides (>15 amino acids) can adopt a helical structure [33], which is strongly predicted for the KALSVETEKLLKYLEAV (216–232) epitope. The extended length of the defined epitopes can also explain why previous epitope scanning of the Ro60KD protein, using synthetic octapeptides, failed to determine these two epitopes [8] whereas study using recombinant fragments of Ro/SSA showed significant antigenicity in the central part of the molecule [9], a finding which correlates well with the present results.

The NGWSHKDLLR and KALSVETEKLLKYLEAV epitopes were also synthesized in their free form and used for inhibition experiments. It was found that the KALSVETEKLLKYLEAV peptide is capable of blocking the antibody activity of the rod bound peptide in a ratio similar to that observed in the case of the Ro60KD antigen. Although the NGWSHKDLLR peptide inhibited antibody binding three times more effectively that the KALVETEKLLKYLEAV peptide, it showed an inhibition ratio approximately half of that caused by the Ro60KD antigen. This finding may derive either from the known enhanced antibody affinity for the pin-bound peptides caused by bivalent binding to the multimeric pin-peptide surface or from partial loss of the activity by length limitation.

Previous studies have shown that one of the major antigenic determinants of Ro60KD is located near the carboxy terminus (485–492) and is homologous to the region of the nucleocapsid N-protein of the Vesicular stomatitis virus (VSV)(8). Testing of this peptide with a panel of autoimmune sera revealed that it was bound only to a limited number of them [34]. These findings have been confirmed in the present study, as no binding was observed in the aforementioned region of the Ro60KD molecule.

The antigenic epitopes presented in this report shared a sequence similarity with the adenovirus E4 TF1 transcription factor, the ribonucleotide reductase of the African swine fever virus and the hypothetical 7·17 protein of *Salmonella choleraesuis.* Although sequence similarities between autoantigens and infective agents can occur by chance, molecular mimicry is still an attractive mechanism that can provide useful information regarding the mechanisms of autoantibody production [35]. In this regard, immunization of rabbits with the N-protein of VSV resulted in the development of a humoral immune response directed towards both Ro60KD protein and VSV [36].

Taking into account that the length of the identified Ro60KD epitopes is rather big, local alignments with other proteins might prove sufficient for possible antibody cross reaction. The NGWSHKDLLR epitope was found to share local identity with the HLA class II β-chain. Surprisingly many of the alleles of the anti-Ro/SSA-associated haplotypes, that is, DR3, DRW53 and DQ2, possess sequence similarities to the NGWSHKDLLR epitope. The DR-related YWNSQKDLLQ sequence, partially homologous

to the NGWSHKDLLR epitope, is localized at a highly accessible site of the HLA DR tertiary structure [37].

The most prominent result of this study is the identification of two discrete B-cell epitopes of the Ro60KD protein selectively recognized from SLE and primary SS patient sera. These findings, as well as the reported sequence similarities between the B-cell epitopes of the Ro60KD protein and other unrelated proteins, may provide clues for the mechanisms responsible for the production of these antibodies. In this regard it is of particular interest that rabbit immunization with short peptides derived from the B/B′ polypeptide of the Sm autoantigen may induce autoantibodies directed against other components of the spliceosome. i.e. the proteins D, 70KD, A and C. Furthermore, the immunized animals developed features of a systemic autoimmune disorder characterized by thrombocytopenia, seizures and proteinuria, as well as antibodies to Sm and to double-stranded DNA [38].

Studies researching the functional immunological role of these epitopes in both *in vitro* and *in vivo* systems are currently under way in our laboratory.

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References

1 Pruijn GJ, Slobbe RL, van Venrooij WJ. Structure and function of La and Ro RNPs. Mol Biol Rep 1990;42:592–600.
2 Pruijn GJ, Wingens PA, Peters SL, Thijssen JP, Van Venrooij WJ. Ro RNP associated Y RNAs are highly conserved among mammals. Biochim. Biophys. Acta 1993;1216:395–401.
3 Tan E. Antinuclear antibodies: Diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol 1989;44:93–151.
4 Naparstek Y and Plotz PH. The role of autoantibodies in autoimmune disease. Ann Rev Immunol 1993;11:79–104.
5 Hartung K, Ehrfeld H, Lakomek HJ, *et al.* The genetic basis of Ro and La antibody formation in Systemic Lupus Erythematosus. Rheumatol Int 1992;11:243–9.
6 Harley JB, Reichlin M, Arnett FC *et al.* Gene interaction at HLA-DQ enhances autoantibody production in primary Sjögren's Syndrome. Science 1986;232:11451–4.
7 Ben-Chetrit EB, Fox RI, Tan EM. Dissociation of immune responses to the SS-A(Ro) 52-kd and 60-kd polypeptides in Systemic Lupus Erythematosus and Sjögren's Syndrome. Arthritis Rheum 1990;33:349–55
8 Scofield RH, Harley JB. Autoantigenicity of Ro/SSA antigen is related to a nucleocapsid protein of Vesicular Stomatitis Virus. Proc Natl Acad Sci USA 1991;88:3343–47
9 Wahren M, Ruden U, Andersson B, Ringertz N, Petterson I. Identification of antigenic regions of the human Ro 60 KD protein using recombinant antigen and synthetic peptides. J Autoimmun 1992;5:319–32.
10 Ricchuiti V, Muller S. Use of peptides for the mapping of B-cell epitopes recognized by antiRo(SSA) antibodies. In: Isenberg DA, Horsfall AC, eds. Autoimmune Diseases: focus on Sjögren's Syndrome London: Bios Scientific Publishers, 1994:101–16.
11 Boire G, Lopez-Longo F, Lapointe S, Menard H. Sera from patients with autoimmune disease recognize conformation determinants on the 60kd Ro/SS-A protein. Arthritis Rheum 1991 34:722–30.
12 Deutscher SL, Harley JB, Keene JD. Molecular analysis of the 60-KDa human Ro Ribonucleoprotein. Proc Natl Acad Sci USA 1988;85:9479–83.
13 Ben-Chetrit E, Gandy BJ, Tan EM, Sullivan KF. Isolation and characterization of a c-DNA clone encoding the 60-KD component of the Human SS-A/Ro ribonucleoprotein autoantigen. J Clin Invest 1989:83;1284–92.
14 Tan EM, Cohen AS, Fries JB, *et al.*The 1982 revised criteria for the classification of Systemic Lupus Erythematosus. Arthritis Rheum 1982;25:1271–77
15 Vitali C, Bombardieri S, Moutsopoulos HM *et al.* Preliminary criteria for the classification of Sjögren's Syndrome. Arthritis Rheum 1993;36:340–7.
16 Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoosd PG. Strategies for epitope analysis using peptide synthesis. J Immun Methods 1987;102:259.
17 Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Society 1963;85:2149–54.
18 Stewart JM, Young JD (eds), Solid Phase Peptide Synthesis. Rockford, I USA: Pierce Chemical Co,1984.
19 Gisin BF. The monitoring of reactions in solid-phase peptide synthesis with picric acid. Anal Chim Acta 1972;58:248–9.
20 Habets WJ, De Rooij DJ, Hoet MH, van de Putte LB, van Venrooij WJ. Quantitation of anti-RNP and anti-Sm antibodies in MCTD and SLE patients by immunobloting. Clin Exp Immunol 1985;59:457-66.
21 Mamula MJ, O'Brien CA, Harley JB, Hardin JA. The Ro Ribonucleoprotein particle; induction of autoantibodies and the detection of Ro RNAs among species. Clin Immunol Immunopathol 1989; 52:435–46.
22 Burkhard R, Sander C. Prediction of protein secondary structure at better than 70% accuracy. J Mol Biol 1993;232:584–99.
23 Pearson WR, Lipman DJ. Improved tools for biological sequence comparison Proc Natl Acad Sci USA 1988;85:2444–8.
24 Smith TF, Waterman MS. Identification of common molecular subsequences. J Mol Biol 1981;147:195–7.
25 Altschul SE. Amino acid substitution matrices from an information theoretic perspective. J Mol Biol 1991;219:555–65.
26 Hopp TP, Wood KR Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci (USA) 198178: 3824–8.
27 Karplus PA, Schulz GE. Prediction of chain flexibility in proteins. Naturwissenschaften 1985;72:212–13.
28 Welling GW, Weijer WJ, van der Zee R, Welling-Westers. Prediction of sequential antigenic regions in protein. FEBS Lett 1985;188:215–18.
29 Elkon KB. Use of synthetic peptides for the detection and quantification of autoantibodies. Mol Biol Rep 1992;16:207–12.
30 Tsuzaka K, Fujii T, Akizuki M *et al.* Clinical significance of antibodies to native or denatured 60-kd or 52-kd Ro/SSA proteins in Sjögren's syndrome. Arthritis Rheum 1994;37:88–92.
31 Habets WJ, Sillekens PT, Hoet MH *et al.* Small nuclear RNA-associated proteins are immunologically related as revealed by mapping of autoimmune reactive B-cell epitopes. Proc Natl Acad Sci USA 1989;86:4674–8.
32 Geyson HM, Mason TJ, Rodda SJ. Cognitive features of antigenic determinants. J Mol Recognit 1988;1:32–41.
33 Kaiser EI, Kezdy FJ. Secondary structures of proteins and peptides in amphiphilic environments. (Review). Proc Natl Acad Sci USA 1983;80:1137–43.
34 Routsias JG, Sakarellos-Daitsiotis M, Detsikas E, Tzioufas AG, Sakarellos C, Moutsopoulos HM. Antibodies to EYRKKMDI Vesicular Stomatitis Virus-related peptide account only for a minority of antiRo60KD antibodies. Clin Exp Immunol 1994; 98:414–18.
35 Horsfall AC. Molecular mimicry and autoantigens in connective tissue diseases. Mol Biol rep 1992;16:139–47.
36 Scofield RH, Huang SC, Pan Z, Harley JB. Induction of anti Ro/SSA by immunization with viral nucleocapsid protein (Abstract) Arthritis Rheum 1994;37:S173.
37 Brown JH, Jardetzky T, Corg JC *et al.*Three dimension structure of

the human class II histocompatibility antigen HLA-DR1. Nature 1993;364:33–9.
38 James JA, Gross T, Scofield RH, Harley JB. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity J Exp Med 1995;181:453–61.