Definition of a Discontinuous Immunodominant Epitope at the NH_2 Terminus of the La/SS-B Ribonucleoprotein Autoantigen

L. Jane McNeilage,* Kandiah Umapathysivam,‡ Elizabeth Macmillan,§‖ Angelo Guidolin,* Senga Whittingham,§ and Tom Gordon*

**Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, South Australia 5042; ‡Rheumatology Research Unit, Repatriation General Hospital, Daw Park, South Australia 5042; §Burnet Clinical Research Unit, The Walter and Eliza Hall Institute, Royal Melbourne Hospital, Victoria 3050, Australia*

Abstract

High-titer IgG autoantibodies to the La/SS-B ribonucleoprotein (RNP) are a hallmark of patients with primary Sjogren's syndrome. Anti-La/SS-B-positive human sera bind to multiple epitopes on recombinant La/SS-B, although the initial response is against an immunodominant epitope within the first 107 NH_2-terminal amino acids (aa). Sequence analysis has identified a striking homology between aa 88-101 in this NH_2-terminal region of La/SS-B and a feline retroviral gag polypeptide suggesting the anti-La/SS-B response may be initiated by cross-reactivity with an exogenous agent. In the present study, detailed mapping of this NH_2-terminal epitope, using recombinant La/SS-B purified from the expression of overlapping DNA fragments spanning aa 1-107, has shown that this immunodominant epitope is a complex conformational or discontinuous epitope dependent upon both aa 12-28 and 82-99 for expression, even though these regions share no homology with each other. This requirement questions the significance of the homology between La/SS-B and a retroviral gag polypeptide in the generation of the B cell response to La/SS-B and is in accord with the general concept that B cells recognize conformational epitopes on antigens rather than small linear peptide sequences. The finding also reinforces the notion that native autoantigen could be the initiator of the autoimmune response. (*J. Clin. Invest.* 1992. 89:1652–1656.) Key words: autoantibody • anti-La/SS-B • epitope mapping • molecular mimicry

Introduction

The application of recombinant DNA technology to the study of autoantibodies to nuclear antigens (ANA)[1] has been an important tool in the analysis of the possible mechanisms of ANA production. The three main theories proposed for autoantibody production have been polyclonal B cell activation (1, 2), molecular mimicry with self (3, 4), and a response initiated and maintained by self (5, 6). While evidence for polyclonal B cell activation exists for some autoantibody responses (1), a number of studies utilizing recombinant nuclear autoantigens (7–16) have provided strong evidence that ANA responses are induced and maintained by self antigen.

There is well established evidence for molecular mimicry between autoantigens and microbial antigens in autoimmune myocarditis. Cross-reactivity has been demonstrated between cardiac antigens and the group A β-hemolytic Streptococcus (17) and cardiac and peripheral nerve antigens and *Trypanosoma cruzi* (18, 19). Molecular mimicry may also account for the induction of ANA. For example, Query and Keene (20) identified an epitope in the 70-kD protein of the U1 small nuclear ribonucleoprotein (snRNP) with homology to the retroviral $p30^{gag}$ protein which they suggested initiated the anti-U1 RNP antibody response. In support of this Cram et al. (15) showed a higher frequency (72% compared with 58%) of antibodies to a recombinant 70-kD U1 RNP protein containing the cross-reactive epitope in newly diagnosed patients with mixed connective tissue disease than in patients with established disease. More recently, Maul et al. (21) identified an epitope on DNA topoisomerase 1 with homology to the retroviral $p30^{gag}$ protein separated by only one amino acid from the region of the $p30^{gag}$ homologous to the 70-kD U1 RNP epitope. A role for virus in the initiation of the autoantibody response to U1 RNP has also been suggested by reactivity of U1 RNP-positive sera with a U1 RNP peptide (ERKRR) with homology to the M1 matrix protein of influenza B viruses (22). Two further studies have highlighted the potential for molecular mimicry in ANA induction. In the first, Kohsaka et al. (23) demonstrated a striking homology (six out of eight consecutive amino acids) between an epitope (aa 88-101) on the La/SS-B ribonucleoprotein (RNP) autoantigen and a viral gag protein. In the second, Garry (24) showed that immunodominant epitopes of the human immunodeficiency virus (HIV) capsid protein are found in regions with homology to epitopes in several autoantigens, including the NH_2-terminal epitope on (aa 88-101) La/SS-B identified by Kohsaka et al. (23). Furthermore, analysis of the reactivity of serial serum samples from three patients with anti-La/SS-B (16) showed that the autoantibody response to La/SS-B was initially directed to an immunodominant NH_2-terminal epitope (aa 1-107) and later over a period of time to other regions of La/SS-B.

The present study examined the nature of the immunodominant NH_2-terminal epitope by employing the polymerase chain reaction (PCR) to generate six overlapping DNA fragments encompassing the NH_2-terminal 107 amino acids of La/SS-B (Fig. 1 *A*). Measurement of the level of binding of a pool of 10 anti-La/SS-B positive sera to the recombinant La/SS-B

Address reprint requests to Dr. L. J. McNeilage, Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, South Australia, 5042, Australia. Elizabeth Macmillian's present address is Hanson Centre for Cancer Research, The Institute of Medical and Veterinary Research, Frome Road, Adelaide, South Australia 5000.

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1. *Abbreviations used in this paper:* aa; amino acid, ANA; antinuclear antibody, GST; glutathione-*S*-transferase, RNP; ribonuclear protein.

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proteins produced by expression of the six DNA fragments demonstrated that two separate regions must be present for binding to occur.

Methods

Derivation of DNA encoding overlapping regions of the N-terminal 107 amino acids of the human La/SS-B autoantigen by PCR. Six DNA fragments encoding the NH_2-terminal 107 aa of the La/SS-B polypeptide were derived by PCR (25). The primers were synthesized on the basis of the published human La/SS-B sequence (26), with the addition of a BamH1 site and an EcoR1 site on the 5′ and 3′ oligonucleotides, respectively. The amplification was performed for 15 cycles, with each cycle being 1.5 min at 95°C, 2 min at 45°C, and 2 min at 72°C. The amplified fragments were purified on low melting-temperature agarose.

Expression of PCR-generated La/SS-B DNA fragments. Each La/SS-B DNA fragment was subcloned into the pGEX-2 vector (27) to generate in-frame fusions with the COOH-terminus of glutathione-*S*-transferase (GST). Transformants were analyzed for recombinant protein production (27), and sequenced to confirm in-frame gene fusions. Recombinant proteins from the six different NH_2-terminal subclones, LaA_1-A_6, were purified (27) and analyzed by SDS-PAGE (28) to verify purity. The recombinant protein bands were identified either by staining the gels with Coomassie blue or by immunoblotting (29) using a polyclonal rabbit anti-GST serum (a gift from C. Davern, The Walter and Eliza Hall Institute for Medical Research). The latter reaction was visualized with a horseradish peroxidase-conjugated sheep anti-rabbit IgG (Silenus, Melbourne, Australia) and enhanced chemiluminescence (Amersham International, Amersham, UK).

DNA sequencing. DNA isolated from the LaA_1-A_6 subclones was sequenced in the pGEX-2 expression vector directly by the dideoxy chain termination method (30) with an alkali-denatured double-stranded template and two primers, designed to anneal 5′ (5′-GCATGGCCTTGCAGGG-3′) and 3′ (5′-CATCACCGAAACGCGC-G-3′), to the pGEX-2 cloning site (16).

Protein determination. The concentration of the purified recombinant La/SS-B-GST proteins was determined by the Lowry protein assay (31) using GST as the standard.

ELISA. Microtiter wells were coated with purified recombinant La/SS-B or GST at a concentration of 0.7 μg/ml in 0.03 M sodium carbonate, pH 9.6, and incubated overnight at 4°C. After blocking for 2 h with 3% BSA in 0.01 M phosphate buffered, 0.15 M saline, pH 7.4 (PBS), the wells were washed three times with PBS-0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO; PBS-Tween) and incubated at 37°C for 1 h with 0.1-ml dilutions of anti-La/SS-B positive sera or sera from healthy controls in 3% BSA-PBS-Tween. After four washes with PBS-Tween, 0.1 ml of an alkaline-phosphate conjugated goat anti-human IgG (Sigma Chemical Co.) was added to each well and incubated for 2 h at 37°C. After four washes with PBS-Tween, each well was incubated with substrate (*p*-nitrophenyl phosphate in diethanolamine, Sigma Chemical Co.) and the optical density at 405 nm (OD_{405}) monitored using a microtiter plate reader (Bio-Rad Laboratories, Richmond, CA).

Measurement of the immunoreactivity of the GST component of each recombinant La/SS-B protein was used to assess its relative adherence to polystyrene wells. Sequential fourfold dilutions of a rabbit polyclonal anti-GST serum were reacted with each of the recombinant La/SS-B-GST proteins, and binding was determined by ELISA using an alkaline phosphatase conjugated anti-rabbit IgG (Sigma Chemical Co.).

In competition ELISAs, sera were first preincubated in the presence or absence of inhibitor for 1 h at room temperature and then added to antigen-coated wells that had been blocked with 3% BSA-PBS. Following a 1-h incubation step, the remainder of the ELISA was performed as described above. The percent inhibition equaled (OD_{405} without inhibitor-OD_{405} with inhibitor)/(OD_{405} without inhibitor) × 100.

Human sera. The 10 anti-La/SS-B positive sera used in this study were derived from the Diagnostic Immunology Laboratory at the Flinders Medical Centre. They were positive for anti-La/SS-B by counterimmunoelectrophoresis and reacted with full-length recombinant La/SS-B (16) by ELISA. 42 sera from healthy individuals were used as controls.

Results

Reactivity of anti-La/SS-B-positive sera with LaA_{1-6} fusion proteins. The 7 regions of the 5′ coding portion of the La/SS-B gene derived by PCR and subcloned into the pGEX-2 expression vector to generate in-frame fusions with GST are defined in Fig. 1 *A*. All 7 subclones produced stable recombinant proteins of the expected M_r with little evidence of degradation as illustrated by Coomassie blue staining (Fig. 1 *B*). The clone containing the LaA_4 fragment produced 5–10-fold more recombinant protein/liter of culture than the other LaA subclones.

Fig. 2 shows a representative immunoblot of the LaA_{1-6} recombinant proteins probed with a polyclonal rabbit anti-GST serum (*A*) and a pool of 10 anti-La/SS-B-positive sera (*B*). The anti-La/SS-B-positive sera reacted only with LaA (aa 1-107), LaA_1 (aa 12-107) and LaA_4 (aa 1-99). Serum from a healthy control did not react with any of the LaA_{1-6} fusion proteins (data not shown).

Table I shows the OD_{405}nm obtained when the 10 anti-La/SS-B positive sera were tested individually for reactivity to the LaA_{1-6} recombinant proteins by ELISA. Among the 10 sera,

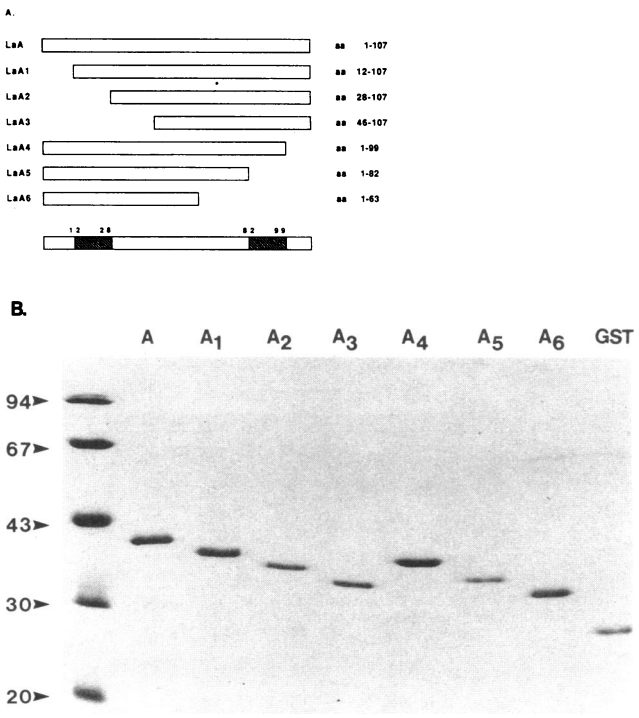

Figure 1. (*A*) Schematic diagram of the PCR-derived LaA and LaA_{1-6} fragments subcloned into the pGEX-2 expression vector (27). The region of the protein encoded by each fragment is given by the amino acid (aa) numbers and the two regions required for expression of the immunodominant epitope within LaA are represented by the shaded areas. (*B*) Coomassie blue stain of the affinity-purified recombinant LaA_{1-6} proteins separated by SDS-PAGE. Molecular weight standards are shown in the first lane.

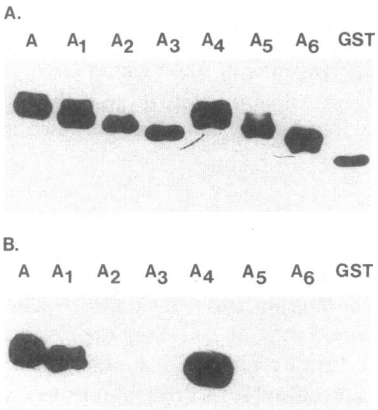

Figure 2. Immunoblot showing reactivity of a polyclonal rabbit anti-GST serum (*A*) and a pool of 10 anti-La/SS-B-positive sera (*B*) with the recombinant LaA proteins and GST.

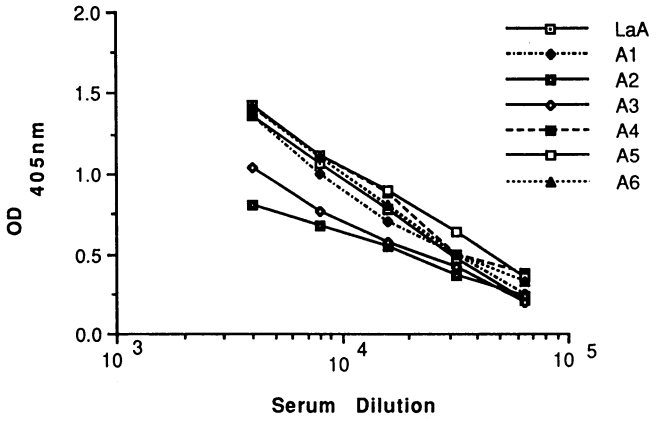

Figure 3. Analysis of recombinant LaA_{1-6} protein adherence to the solid phase by reactivity with a polyclonal rabbit anti-GST serum. Each of the recombinant LaA_{1-6} proteins was coated onto microtiter wells at 0.7 μg/ml and reacted by ELISA with sequential dilutions of the rabbit anti-GST serum. The binding of the rabbit anti-GST serum to the recombinant proteins is given by the OD_{405}nm.

the mean optical densities (OD_{405}nm ± standard deviation) were: LaA, 1.27 (±0.29); LaA_1 1.10 (±0.21); LaA_2 0.003 (±0.001); LaA_3 0.01 (±0.01); LaA_4 1.19 (±0.33); LaA_5, 0.01 (±0.01); and LaA_6 0.01 (±0.01). The mean OD_{405}nm values of the anti-La/SS-B-positive sera were significantly greater than controls reacted with recombinant LaA_1 and LaA_4 but did not differ significantly from controls reacted with recombinant LaA_2, A_3, A_5, or A_6. The ELISA results for the individual anti-La/SS-B-positive sera confirm the pattern of reactivity seen on the immunoblot (Fig. 2 *B*) with the pooled anti-La/SS-B-positive sera.

The direct comparison of levels of antibody binding to the different LaA_{1-6} recombinant proteins assumes adherence of each to the solid phase. This was tested by measuring the immunoreactivity of the GST component of each recombinant protein. At an antigen coating concentration of 0.7 μg/ml sequential dilutions of a polyclonal rabbit anti-GST serum reacted strongly with LaA and each of the recombinant LaA_{1-6} proteins although binding to LaA_2 and LaA_3 was lower than to the other recombinant proteins (Fig. 3). Despite doubling of the coating concentration for LaA_2 and LaA_3 the pooled anti-La/SS-B-positive sera still failed to react with either of these recombinant proteins (data not shown). These results suggest that each of the LaA subfragments adhered effectively to microtiter wells and that failure to detect binding of anti-La/SS-B positive sera to the $LaA_{2,3,5}$ and A_6 subfragments was not due to poor coating of antigen.

Competitive inhibition of antibody binding to LaA by the LaA_1 and LaA_4 subfragments. Preincubation of the pooled anti-La/SS-B-positive sera with different concentrations of recombinant LaA, LaA_1 and LaA_4 proteins significantly reduced the level of anti-La/SS-B binding to LaA (Table II). In contrast,

Table I. Direct Binding of Anti-La/SS-B-positive Sera to Recombinant LaA_{1-6} by ELISA

	Binding (expressed as OD_{405} nm) to						
Patients	LaA	LaA_1	LaA_2	LaA_3	LaA_4	LaA_5	LaA_6
1	1.06	0.66	0.01	0.01	0.83	0.03	0
2	1.28	1.17	0.01	0.02	1.15	0.09	0.04
3	1.39	1.21	0	0.01	1.52	0.01	0
4	1.16	0.87	0	0	0.72	0.03	0.01
5	1.43	1.21	0	0	1.29	0.02	0
6	1.43	1.21	0	0	1.12	0.04	0
7	0.75	1.17	0	0.01	1.06	0	0
8	1.85	1.35	0.01	0.01	1.90	0	0
9	1.06	0.97	0	0	1.15	0.02	0.02
10	1.29	1.21	0	0	1.16	0.04	0.01
Mean	1.27	1.10	0.003	0.01	1.19	0.01	0.01
SD	0.29	0.21	0.001	0.01	0.33	0.01	0.01
Healthy controls							
Mean	0.02	0.04	0.02	0.05	0.03	0.10	0.08
SD	0.01	0.03	0.02	0.03	0.01	0.05	0.06

Table II. Inhibition of Anti-La/SS-B Binding to Recombinant LaA Proteins

		% Reduction in anti-LA/SS-B binding to		
Inhibitor*	mg/ml	LaA	LaA_1	LaA_4
LaA_1	10	99	98	99
	1	99	96	97
	0.1	96	42	75
	0.01	49	13	36
LaA_4	10	100	96	99
	1	85	58	84
	0.1	52	16	33
	0.01	42	10	16
LaA_3	10	34	NT§	NT
	1	30	NT	NT
	0.1	24	NT	NT
	0.01	30	NT	NT
GST‡	10	24	NT	NT
	1	20	NT	NT
	0.1	24	NT	NT
	0.01	24	NT	NT

* The pooled anti-La/SS-B sera were incubated with different concentrations of inhibitors for 1 h at room temperature. ‡ A similar reduction in binding was seen with recombinant LaA_2, LaA_5, and LaA_6. § NT, not tested.

preincubation of the pooled sera with different concentrations of recombinant LaA_2, LaA_3, LaA_5, and LaA_6 proteins failed to substantially alter the level of anti-La/SS-B binding to LaA (Table II). A lack of inhibition by GST alone (Table II) confirms that antibodies in the anti-La/SS-B positive sera react with sequences in the La/SS-B portion of the recombinant proteins and not with the GST moiety.

Discussion

This study has used recombinant human La/SS-B proteins comprising six overlapping regions of the NH_2-terminal 107 amino acids of the La/SS-B autoantigen to further define the immunodominant epitope contained within this region of La/SS-B. This data is in apparent contrast to that obtained by Kohsaka et al. (23) who mapped the immunodominant NH_2-terminal epitope to amino acids 88-101. However, Kohsaka et al. (23) used overlapping fragments derived only by 3′ deletions of the La/SS-B cDNA, in contrast to the overlapping fragments used in the present study which contained both 5′ and 3′ deletions. Use of recombinant proteins expressed by subfragments LaA_1, LaA_2, and LaA_3, all of which contain 5′ deletions (Fig. 1 *A*), has revealed the requirement for amino acids 12-28 in formation of the NH_2-terminal epitope. This is supported by data from an earlier study (9) showing that a La/SS-B fragment encoding amino acids 54-137 was not immunoreactive. Furthermore anti-La/SS-B binding was detected to only two of the six recombinant proteins, LaA_1 (aa 12-107) and LaA_4 (aa 1-99), and these were the only two proteins to inhibit binding to recombinant LaA (aa 1-107). These data, together with the fact that the two regions, amino acids 12-28 and 82-99, are only present in LaA_1 and LaA_4, suggest that the immunodominant NH_2-terminal epitope on La/SS-B is a discontinuous or conformational epitope requiring amino acids 12-28 and 82-99 for expression.

Identification of a discontinuous or conformation-dependent epitope in the region of La/SS-B to which the anti-La/SS-B response may be initiated (16) raises questions about the significance of the homology identified (23) between amino acids 88-101 of La/SS-B and amino acids 22-50 of a feline sarcoma virus gag polypeptide. It would seem unlikely that an isolated region of sequence similarity in a viral gag protein could mimic a complex conformational determinant involving disparate regions of a self antigen. However, whereas the data imply that anti-La/SS-B recognizes the retroviral sequence in La alone it may also recognize that sequence in conjunction with a different region on the retrovirus. This implies that the two hypotheses for the generation of autoantibodies, molecular mimicry and anti-self may not be mutually exclusive. The demonstration of direct binding of anti-La/SS-B to the relevant retroviral protein will be important in further elucidation of these mechanisms. Furthermore, it cannot be discounted that such linear regions of similarity between self and viral antigens may be of more importance in the loss of T cell tolerance to autoantigens. Homologies between epitopes on other nuclear autoantigens and viral proteins have been reported previously (20–22, 24) and while it is possible that infection with a virus could induce the production of antibodies that react with a limited number of autoepitopes, this mechanism does not entirely explain the generation of an autoimmune response that is directed against multiple epitopes on the nuclear autoantigen (7–16), some of which are conformational. Indeed conformation-dependent epitopes have been identified on a number of autoantigens (14, 32–36) and may be formed by amino acid residues from two or more regions of the molecule being brought into proximity to one another by protein folding (36). It should be noted that Geysen et al. (37) have shown that a linear hexapeptide with the correct stereochemistry could substitute for an epitope composed of three separate regions of a foot and mouth disease viral antigen. A similar analysis for the immunodominant NH_2-terminal La epitope will be important in further defining reactivity to this epitope.

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