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Epitope mapping of the 52-kD Ro/SSA autoantigen

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SUMMARY

Autoantibodies to Ro/SSA are commonly found in sera of patients with systemic lupus erythematosus (SLE) and primary Sjögren's syndrome. The presence of these antibodies is related to lymphopenia, photosensitive dermatitis, and pulmonary and renal disease, suggesting that they have an immunopathologic role [1–6]. We previously isolated a cDNA clone which encodes the 52-kD human Ro/SSA protein. In this study we have determined the number and location of epitopes recognized by SLE sera using recombinant proteins encoded by the full-length or overlapping subclones of this cDNA. An immunodominant epitope was detected using Western blots and ELISA on the NH_2-terminal side of this protein's putative leucine zipper. The data suggest that 11 amino acids are critical for the recognition of this molecule by these autoantibodies. Although the titres of anti-52-kD Ro/SSA antibodies vary between different patient sera, no heterogeneity in the location of antigenic epitopes to which their autoantibodies bound was detected. This homogeneous pattern of reactivity to a single rather than multiple regions of this protein is unusual for lupus autoantigens which have been identified, and suggests that these antibodies may have arisen as by a cross-reaction to an epitope on another molecule.

Keywords autoimmunity Ro/SSA systemic lupus erythematosus autoantibodies

INTRODUCTION

Biochemical characterization of the Ro/SSA autoantigen has revealed significant heterogeneity in both the protein and RNA components of this particle. Two families of Ro/SSA proteins have been described. Anti-Ro/SSA autoantibodies bind to a 60-kD protein which is present in mammalian nucleated cells and primate erythrocytes [7,8]. This protein is non-covalently associated with one of four small RNA molecules between 83 and 112 nucleotides in length [9–13]. Approximately 70% of the patients with antibodies to the denatured 60-kD Ro/SSA protein also have antibodies which bind to a 52-kD protein [8,14], and all of the patients with these anti-52-kD antibodies have antibodies which bind to the native 60-kD Ro/SSA complex. This 52-kD Ro/SSA protein is present in nucleated cells and platelets [15]. Antibodies which bind to this 52-kD protein cross-react with a 54-kD protein in erythrocytes and their nucleated precursors in Western blots, but not to the 60-kD Ro/SSA protein. Similarly, antibodies eluted from the 60-kD proteins of Western blots fail to recognize the 52-kD or 54-kD proteins. Therefore, Ro/SSA polypeptides have been grouped into two non-cross-reacting families [8].

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To define better the relationship between the 60-kD and 52-kD forms of the Ro/SSA protein, we previously cloned and determined the sequence of cDNA of the 52-kD protein's gene [16]. A cDNA sequence which differs in a single replacement substitution in the coding region was simultaneously reported by Chan *et al.* [17]. Both sequences contain an open reading frame which encodes a predicted full-length polypeptide of 475 amino acids. Based on this amino acid sequence, the 52-kD protein is expected to contain both a zinc finger and leucine zipper structure. The lack of similarity between the 60-kD and 52-kD Ro/SSA sequences is consistent with the absence of immunological identity described above [18,19].

Diversity has been recognized not only in the antigens recognized by Ro precipitin-positive sera, but also in the patterns of antibodies present in such sera. Itoh & Reichlin [20] determined that the majority of Ro precipitin-positive sera contained antibodies to the denatured 52-kD Ro/SSA protein. These antibodies were present in sera containing antibodies which bound the native 60-kD Ro/SSA complex, as well as in sera with both anti-60-kD Ro/SSA and anti-La/SSB antibodies. Only the latter sera, which represent approximately 20% of systemic lupus erythematosus (SLE) sera contain antibodies which immunoprecipitated the native 52-kD Ro/SSA protein. Therefore, autoimmune responses to the 52-kD Ro/SSA can be directed to epitopes on the denatured and the native molecule. Interestingly, no antibodies to either form of 52-kD Ro/SSA were identified in sera with Ro/SSA and U1-RNP precipitins.

In this study we have analysed the autoepitopes on the 52-kD Ro/SSA protein through deletion mapping analysis of recombinant proteins expressed in *Escherichia coli*. A single immunodominant region on the denatured protein was localized to the putative leucine zipper. All patient sera tested which have antibodies that bind to the 52-kD protein in Western blots have antibodies which recognize this region, suggesting that this linear epitope is essential for the antigenicity of this protein. The observation of an autoimmune response to a single region of an antigen is unusual among the epitopes which have been defined on lupus antigens. No relationship between this epitope and the 60-kD Ro/SSA protein was found, consistent with the lack of cross-reactivity of antibodies which bind to determinants of these denatured molecules.

PATIENTS AND METHODS

Sera

Sera from 36 patients who satisfied the American Rheumatism Association criteria for SLE [21], plus eight unrelated healthy controls were kindly provided by Dr Morris Reichlin (Oklahoma Medical Research Foundation). Affinity-purified rabbit anti-GST antibodies were kindly provided by Dr Steven Taffet (Department of Microbiology & Immunology, S.U.N.Y. Health Sciences Centre, Syracuse, NY).

Expression and epitope mapping of the recombinant 52-kD Ro/SSA proteins

The whole 52-kD Ro/SSA cDNA was subcloned into the pUC18 plasmid and transformed into *E. coli* strain DH5α (clone A). This clone contains the entire 1425-bp open reading frame of the human 52-kD Ro/SSA cDNA. Five overlapping regions of this cDNA were generated by digestion of the cDNA with restriction endonucleases, and were cloned either downstream of the lac promoter in pUC vectors, or ligated in-frame to the glutathione S-transferase (GST) gene of *Schistosoma japonicum* for bacterial expression. The restriction enzymes used for isolation of portions of the cDNA and their location on the cDNA in parentheses were *Eco*RI(1) to *Pvu*II(626) for clone B, *Eco*RI(1) to *Sau*3AI(734) for clone C, *Rsa*I(441) to *Sau*3AI(734) for clone D, *Pvu*II(659) to *Bam*HI(911) for clone E, and *Alu*I(751) to *Alu*I(1593) for clone F. Double-stranded DNA from these clones was denatured in NaOH [22] and sequenced using the dideoxy chain-termination method [23] with T7 DNA polymerase [24] to confirm the orientation and proper reading frame of all cDNA inserts. Furthermore, the presence of the appropriate sized proteins encoded by each of these clones was confirmed in Western blots using lupus patient sera or affinity-purified rabbit anti-GST antisera.

For immunoblot assays, bacterial cells were boiled in 2× sample buffer (130 mM Tris-HCl pH 6·8, 10% 2-mercaptoethanol, 20% glycerol, 4% SDS, and 0·001% bromophenol blue). Ten per cent and 12·5% SDS–polyacrylamide gels were used for size separation of the full length 52-kD protein or for its subregions, respectively. Protein samples were electroblotted (MilliBlot-SDE; Millipore Corp., Bedford, MA) onto nitrocellulose membranes (BioBlot-NC; Costar, Cambridge, MA) and stained with 0·1% fast green in 10% (v/v) acetic acid and 20% (v/v) methanol. Membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris-HCl pH 8·0, 150 mM NaCl, 0·05% Tween 20) and exposed to sera diluted 1:100 or 1:1000 in TBST with gentle agitation. Following four washes in TBST, membranes were exposed to goat anti-human IgG (for use with patient sera) or goat anti-rabbit IgG (for use with the anti-GST antibodies) alkaline-phosphatase conjugated antibodies (1:5000 dilution; Sigma Chemical Co., St Louis, MO). Following TBST washes, the reactions were visually monitored with the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Kirkegaard & Perry Labs, Gaithersburg, MD).

An ELISA assay for the detection of the 52-kD Ro/SSA recombinant protein and its immunoreactive subregions was developed. Transformed *E. coli* were resuspended in lysis buffer (0·1 M NaCl, 50 mM Tris-HCl pH 8·0, 10 mM EDTA) containing fresh 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell walls were digested with lysozyme (10 mg/ml in 0·25 M Tris-HCl) and spheroblasts were disrupted by sonication. Insoluble recombinant proteins were pelleted by centrifugation at 10 000 ***g***, 4°C for 15 min. The pellets were solubilized in 7 M urea and diluted in carbonate coating buffer (0·012 M Na_2CO_3, 0·167 M $NaHCO_3$, 0·001 M $MgCl_2$, pH 9·6) before use in direct binding or antibody blocking experiments. A standard protocol for ELISA in 96-well microtitre plates (Costar) was used [25]. Serial 10-fold dilutions of serum ranging from 10^{-1} to 10^{-6} were studied. Reactivity of alkaline phosphatase-conjugated goat anti-human IgG with 1 mg/ml *p*-nitrophenyl phosphate in carbonate substrate buffer (carbonate coating buffer pH 8·7) was monitored at 405 nm with a Microelisa Reader (Dynatech, Alexandria, VA). The serum dilution of patient 1 which produced 50% of the maximum level of binding in ELISA was arbitrarily defined as 500 U. This dilution corresponded to $2{\cdot}6 \times 10^{-4}$. Unit values for other patients' sera were calculated relative to this standard reference sera following subtraction of antibody reactivity to *E. coli* proteins transfected with an expression vector which lacked the human 52-kD Ro/SSA cDNA insert.

Oligopeptide synthesis

Two oligopeptides were prepared by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Centre using the Fmoc synthesis strategy [26]. The names, origin, and amino acid sequences of these oligopeptides are 'Peptide 1' (52-kD protein, residues 195–209): Arg-Gln-Leu-Gln-Glu-Leu-Glu-Lys-Asp-Glu-Arg-Glu-Gln-Leu-Arg; 'Peptide 2' (52-kD protein, randomized order of residues 195–209): Leu-Asp-Glu-Gln-Leu-Gln-Glu-Arg-Arg-Glu-Leu-Gln-Arg-Glu-Lys.

Computer analysis

Searches for similarities to sequences in existing nucleic acid and protein computer databases were performed using the Sequence Analysis Software Package of the Genetics Computer Group [27], the Blast network service of the National Center for Biotechnology Information [28], and programs developed for use at the Pittsburgh Supercomputer Facility.

RESULTS

Expression of a human recombinant 52-kD Ro/SSA protein in E. coli

The 52-kD Ro/SSA cDNA was originally cloned in our laboratory from a lambda-gt11 library. We expressed this gene's product as an IPTG-induced β-galactosidase fusion protein in

this bacteriophage vector. A specific band was detected using an SLE patient's serum (patient 1) which contained antibodies which bind to a 52-kD region in Western blots of size-separated human cell extracts [16].

In an attempt to enhance the yield and develop a sensitive ELISA to detect anti-52-kD Ro/SSA antibodies, this cDNA was subcloned into the pUC19 plasmid vector [29] and expressed in *E. coli* strains DH5α, PR13Q, and JM109. The recombinant protein was immunoreactive with sera known to contain anti-52-kD Ro/SSA antibodies, as well as with affinity-purified antibodies eluted from the 52-kD region of human lymphocyte extracts separated on polyacrylamide gels. A predominant 56-kD recombinant fusion protein, along with faint 42-kD and 35-kD bands, was detected with the patient antibodies (Fig. 1). This size is consistent with the predicted molecular mass of 56 920 D from the full-length 52-kD Ro/SSA protein plus 25 residues on the NH_2-terminal end which are encoded by the pUC19 vector. The presence of the two smaller bands was correlated with the use of aged stocks of the fusion protein, suggesting that these represent partially degraded products of the full-length protein. Antibodies in normal human sera failed to bind to the recombinant protein. No differences in expression related to the use of different bacterial strains were found, and recombinant products expressed in *E. coli* strain DH5α will be described in the remainder of this study.

An ELISA was developed to detect antibodies which react with this recombinant protein. *Escherichia coli* lysates from bacteria transformed with pUC19 plasmids containing the whole cDNA were used to coat 96-well microtitre plates. As negative controls, other wells were coated with no antigen (i.e. urea diluted in coating buffer), and with identically treated extracts from *E. coli* transformed with pUC19 containing no insert. Sera from 36 SLE patients, half of whom had anti-52-kD Ro/SSA antibodies detectable in Western blots using the human cell line Molt-4 as a source of antigen, and eight healthy controls were screened with these extracts. Seventeen of the 18 patients with anti-52-kD antibodies detectable in Western blots reacted positively with the recombinant protein. Fifty per cent maximal activity of these sera was detected at serum dilutions between 2×10^{-3} and 6×10^{-7}. Among these sera, the activity varied over two orders of magnitude (Fig. 2). The anti-52-kD antibody titres were independent of the presence of Western blot-detected anti-60-kD Ro/SSA autoantibodies. As expected, this ELISA was more sensitive for detecting anti-52-kD autoantibodies than Western blots. Patient sera with no apparent anti-52-kD antibodies detectable with human cell lysates or the recombinant protein in Western blots fell into two groups. Twelve of 18 patient sera had no detectable reactivity to the recombinant protein in ELISA, while six patient sera had antibody titres at least an order of magnitude less than the patients with Western blot-detectable anti-52-kD antibodies. Antibodies from three of these six patients were blocked with the whole recombinant protein, while antibodies from the other three patients (Fig. 2, open circles) showed no evidence of blocking, indicating a non-specific reactivity. The autoantibody titres determined in ELISA were highly correlated with a semiquantitative score of the intensity of the denatured 52-kD antigen on Western blots ($r = 0{\cdot}84$; $P < 10^{-5}$), but not with the presence of anti-60-kD Ro/SSA, -La/SSB, or -nRNP autoantibodies. Antibodies from all healthy controls tested failed to react with the recombinant protein.

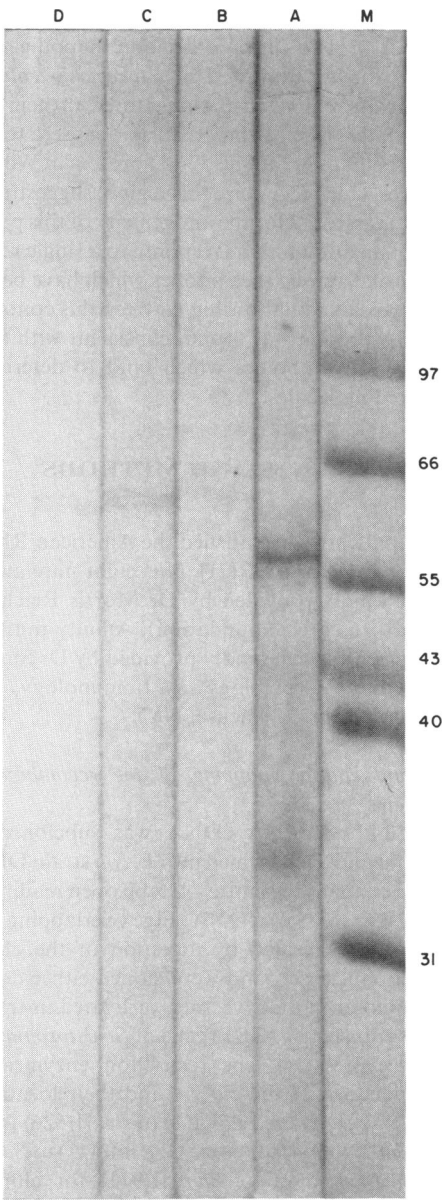

Fig. 1. Western blot reactivity of antibodies to the human 52-kD Ro/SSA recombinant protein. Extracts from *Escherichia coli* transformed with a plasmid containing the whole cDNA for the 52-kD gene (lanes A and B), or non-recombinant pUC19 plasmids (lanes C and D) were separated and transferred to nitrocellulose membranes. Serum from a patient with antibodies which bind to the 52-kD human protein (lanes A and C), or normal human serum (lanes B and D) were used to detect the presence and size of immunoreactive proteins. Molecular weight standards ($\times 10^{-3}$ kD) are listed in lane M.

Epitope mapping of the 52-kD Ro/SSA protein

Because antigenic activity was present in the recombinant 52-kD Ro/SSA protein, we designed experiments to map the location of the epitopes on these molecules. The full-length cDNA was digested with restriction enzymes to produce overlapping regions which were subcloned into expression vectors. Five different clones were produced (Fig. 3B–F). DNA from each clone was sequenced to verify in-frame ligations to the vectors, and the presence of the appropriate sized fusion

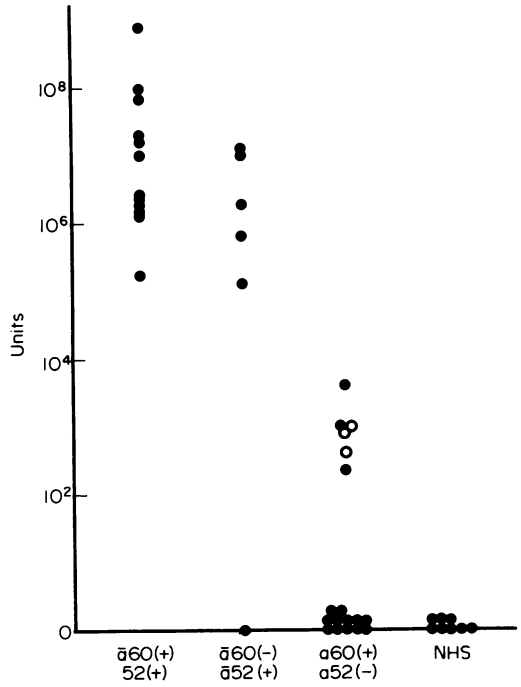

Fig. 2. Anti-52-kD Ro/SSA autoantibody activity. Patients were divided into three groups according to the presence or absence of antibodies (detected with Molt-4 cell extracts in Western blots) to the 60-kD and 52-kD Ro/SSA proteins. NHS, Normal human sera. o, Non-specific binding activity of antibodies in a subset of patients which was not blocked by the recombinant protein.

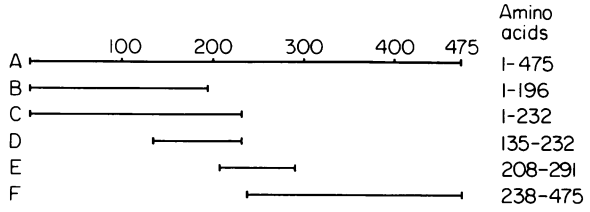

Fig. 3. Subclones of the 52-kD Ro/SSA cDNA. Six clones designated A–F were produced. The amino acid residues of the 52-kD Ro/SSA protein expressed by these clones are shown to the right.

proteins were confirmed in Western blots. The recombinant proteins produced by these deletion mutants were analysed in both Western blots and ELISA, initially using patient 1 serum which has antibodies reactive in Western blots with only the human 52-kD Ro/SSA protein. In ELISA and Western blot assays, proteins expressed only by clones A, C and D reacted with these antibodies, but not with antibodies present in normal human serum (Figs 1 and 4).

Blocking experiments using recombinant proteins expressed by these immunoreactive clones were performed. Extracts from lysed Molt-4 cells were separated by 10% SDS–PAGE, transferred to nitrocellulose membranes and reacted with serum from patient 1. No diminution of binding activity was observed when proteins expressed by *E. coli* transformed with a pUC19 vector lacking the 52-kD Ro/SSA cDNA insert were used to block reactivity (Fig. 5). However, complete blocking of 10^{-2} and 10^{-3} serum dilutions was observed when using proteins expressed from the whole cDNA insert (clone A), and from clone D.

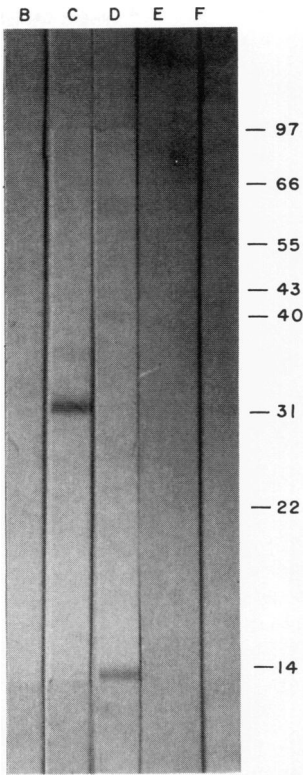

Fig. 4. Western blot of recombinant proteins expressed by the 52-kD Ro/SSA cDNA deletion clones. A systemic lupus erythematosus (SLE) patient serum with anti-52-kD Ro/SSA activity was used for detection of antigen. No antigen was detected with normal human sera. Molecular weight markers are shown to the right ($\times 10^{-3}$ kD).

Proteins from clone C totally blocked reactivity of a 10^{-3} serum dilution, and blocked approximately 80% of the reactivity of the 10^{-2} serum dilution. No blocking was observed when proteins expressed from clone B or F. Molt-4 cell extracts were similarly capable of blocking reactivity to the recombinant 52-kD Ro/SSA protein in Western blots (data not shown). These data suggest that a linear epitope located adjacent to the leucine zipper motif which spans residues 197–232 of this protein plays an important role in the antigenicity of this molecule.

In a direct binding ELISA, proteins expressed by clone C reacted with patient 1 antibodies to the same extent as proteins produced by the whole cDNA. The reactivity of recombinant proteins expressed by clone D was only slightly lower. No reactivity with proteins expressed by the other deletion mutants was detected. The reactions with proteins of clones C and D, but not B, suggest that amino acids to the COOH-terminal side of residue 196 (Fig. 3) are of critical importance to the antigenic structure of this protein. The absence of reactivity to recombinant proteins expressed by clones E and F further narrows this region to amino acid residues 197–207 of the full-length protein. This region corresponds to the NH_2-terminal portion of the 52-kD Ro/SSA putative leucine zipper [16].

The initial mapping of this epitope was performed using serum from a single lupus patient. In order to study the possible heterogeneity of antibody binding to other regions, sera from a total of 21 anti-52-kD Ro/SSA-positive patients (five of which had anti-La antibodies, seven of which did not, and two of

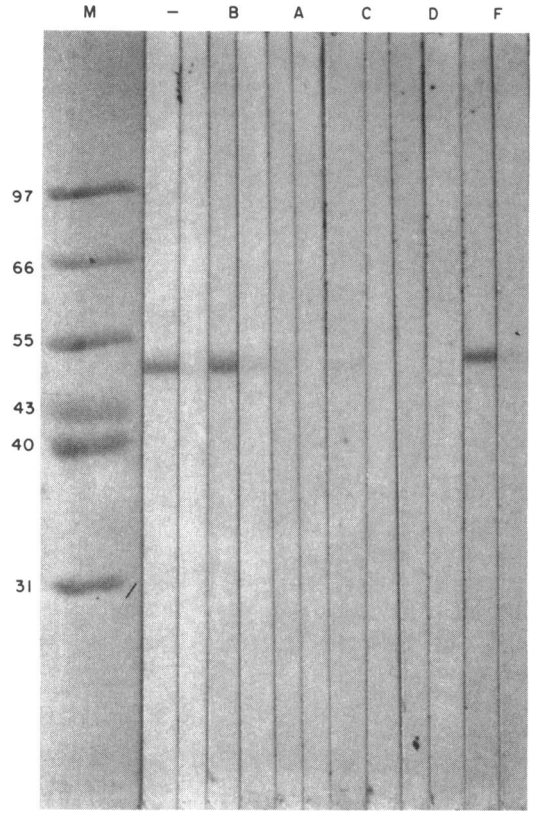

Fig. 5. Western blot of the human 52-kD Ro/SSA protein using serum blocked with extracts of the 52-kD Ro/SSA clones. Molt-4 extracts were transferred to nylon membranes in duplicate as a source of antigen. Sera diluted 10^{-2} (left section of each pair) and 10^{-3} (right section) were used either unadsorbed ('–' lane) or adsorbed with recombinant proteins produced by the various clones of the 52-kD Ro/SSA cDNA. Serum used in this experiment was from patient 1.

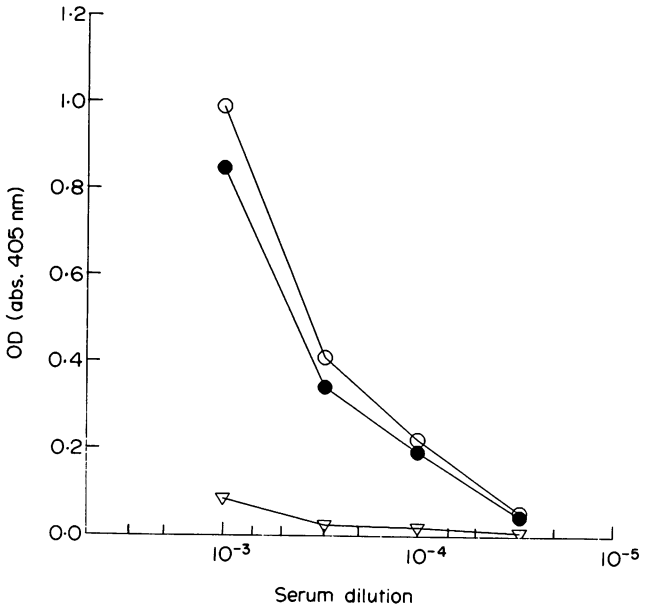

Fig. 6. ELISA of the human recombinant 52-kD Ro/SSA protein using serum blocked with oligopeptides. ○, No blocking; ▽, blocking with oligopeptide 1, ●, blocking with oligopeptide 2. The patient serum used for this experiment is the same as that used for Fig. 5.

which had anti-nRNP antibodies) plus five healthy controls were tested for reactivity with proteins expressed by these deletion mutants in ELISA. Every patient's serum reacted only with products from clones C and D. No reactivity was detected using serum obtained from healthy individuals. No sera were found which reacted with proteins expressed by the other clones. Thus, no evidence for heterogeneity in the binding sites for autoantibodies from different SLE patients to this protein was detected. These results support the hypothesis that the 52-kD Ro/SSA antigen has an immunodominant epitope immediately adjacent to the leucine zipper motif which is recognized by antibodies in lupus patient sera with anti-52-kD Ro/SSA activity.

In order to confirm that this region represents the immunodominant epitope of the 52-kD Ro/SSA autoantigen, two oligopeptides were synthesized and used as blocking reagents for antibody binding to the recombinant protein in ELISA or to the 52-kD Ro/SSA protein of Molt-4 cells in Western blots. Peptide 1 contained the amino acid residues identified as the epitope in the deletion experiments described above (residues 197–207) plus the two amino acids on each side of this region. Peptide 2 consisted of a randomized sequence of that present in peptide 1, and was used as a control for amino acid composition *versus* sequence-specific binding. In ELISA, peptides 1 and 2 blocked 92% and 14%, respectively, of the binding of antibodies to the full-length recombinant protein (Fig. 6). Similarly in Western blots, all reactivity of a 10^{-3} dilution of serum was blocked with oligopeptide 1, with no attenuation of binding when using serum adsorbed with oligopeptide 2 (data not shown). These antibody blocking patterns are consistent with the reactivity of SLE patient autoantibodies to fusion proteins expressed by clones C and D, but not those of clones B and E. These results confirm the existence of an immunodominant autoepitope on the NH_2-terminal portion of the leucine zipper motif of the 52-kD Ro/SSA protein.

DISCUSSION

Although anti-52-kD Ro/SSA autoantibodies are frequently detected in lupus sera at very high dilutions, the number of epitopes to which they are directed is quite small. Our data indicate the presence of a single epitopic region on the NH_2-terminal side of the putative leucine zipper. Anti-52-kD Ro/SSA-positive sera from SLE patients reacted only with the recombinant proteins produced from cDNA inserts which contain the region encoding this portion of the leucine zipper. These recombinant proteins as well as an oligopeptide identified from the deletion cloning experiments blocked the reactivity of patient antibodies to the 52-kD Ro/SSA protein. Identical blocking was observed using extracts from HeLa cells and normal human peripheral blood leucocytes (Frank and Itoh, unpublished observations). No evidence for reactivity to another epitope was detected in serum from 21 patients studied, suggesting a lack of heterogeneity of the antibody binding site of this protein. The strong correlation between the antigenic activity observed with Western blots and the ELISA immunoreactivity strongly suggests that the latter assay detects antibodies directed primarily to a denatured epitope.

Recent observations indicate that the 52-kD Ro/SSA protein in human cells is not immunoprecipitated with sera

containing anti-52-kD antibodies (Y. Itoh *et al.*, manuscript in preparation). This lack of reactivity to the native particle may be explained if the sole epitope of this protein is present on a functional leucine zipper involved in protein dimerization. In this case, one would predict these leucine zipper residues would not be exposed to solvent and would become immunoreactive only after denaturation.

Epitope mapping of other human autoantigens including La/SSB [30–32], proliferating cell nuclear antigen [33], Sm-B′/B [34], hystidyl-tRNA synthetase [35], U1RNP [36], and the 60-kD Ro/SSA protein [37] has been reported. In each of these studies, variation in autoantibody binding to multiple epitopes of these proteins has been found. Studies of anti-histone antibody responses by lupus patients have revealed a more limited distribution of epitopes [38,39]. SLE anti-H2A antibodies bound to both the amino and carboxyl termini of this protein [40]. Most anti-H1 antibodies bound to the COOH-terminal region, although weak responses to an NH_2-terminal epitope were also seen [38]. This pattern is similar to that of myasthenia gravis patients' anti-acetylcholine receptor (AcChR) response, where the majority of such antibodies were found to bind to a 'main immunogenic region' of AcChR, yet other portions of the molecule were immunologically recognized as well [41]. While lupus autoantibodies binding to histone H2B are restricted to the NH_2-terminus, evidence for heterogeneity in this system has also been observed [38]. Similar mapping of the NH_2-terminal epitope of H2B in a murine model of SLE showed that antibodies present in 15% of anti-H2B-positive animals were not blocked with an amino-terminal peptide derived from the immunodominant portion of this protein, suggesting that the heterogeneity in the antibody response to this conserved epitope is not limited to humans [42].

We are aware of only one other study which reported the presence of a single epitope on a denatured lupus autoantigen. Elkon *et al.* showed that autoantibodies binding to the ribosomal P proteins in Western blots were restricted to the COOH-terminus and could be totally blocked by a highly conserved 22 amino acid polypeptide [43]. More recent studies using a sensitive ELISA have confirmed this observation [44]. It thus appears that the finding reported here of a lupus immune response to a single epitope on the denatured 52-kD Ro/SSA protein is unusual. Whether this is representative of a distinct immune response to a limited portion of this protein or a cross-reaction of these autoantibodies which are produced in response to a different antigen remains to be determined. This study does not address the possibility of additional epitopes which may be present on the native 52-kD Ro/SSA protein.

Studies of epitopes of the 60-kD Ro/SSA protein have revealed areas of similarity to the Indiana strain of the vesicular stomatitis viral nucleocapsid N protein [37]. While the absence of this particular virus in Europe precludes it from being the etiological agent of lupus, the virus is a member of an extensive family, one or more of which may have importance in disease pathogenesis. Computer searches for similarities between the epitope of the 52-kD Ro/SSA protein and proteins in the NBRF and SWISSPROT databases revealed similarities to very short regions of charged residues present in a number of unrelated proteins. None of these similarities has produced insights into what the initial immunogen which gave rise to these autoantibodies may be. However, the number of proteins in these databases remains small. The data presented here demonstrate that human autoimmune responses may be directed against very limited portions of particular molecules. The definition of such regions could have important implications in disease pathogenesis and for future experimental designs concerning etiological agents of systemic lupus erythematosus.

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