

Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus

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ABSTRACT The characteristics of eukaryotic ribosomal proteins P0, P1, and P2 (P proteins) and their antigenic determinants were studied using the sera of patients with systemic lupus erythematosus (SLE). P0, P1, and P2 were isolated as a macromolecular complex by preparative isoelectric focusing and anion-exchange chromatography in the presence of 6 M urea. The apparent molecular size of the complex was 140 kDa as determined by gel filtration on a Sephadex G-200 column. P0 may, therefore, be the eukaryotic equivalent of *Escherichia coli* ribosomal protein L10. In addition, all three P proteins were detected in the postribosomal supernatant of HeLa cells, and P0 and P1 were found to be more acidic than their ribosome-bound counterparts. Partial proteolysis experiments revealed that SLE anti-P sera recognized one or both ends of the P2 equivalent protein from *Artemia salina* (eL12). Sixteen SLE sera containing antibodies to P0, P1, and P2 reacted with a carboxyl-terminal peptide 22 amino acids in length of eL12 and not with an amino-terminal peptide of 20 amino acids. Even though the carboxyl-terminal peptide completely inhibited the ability of the antiserum to react with all three proteins on an immunological blot, the same peptide produced only small decreases in binding of the SLE antibody to the native, nondenatured P proteins. These findings indicate that SLE anti-P antibodies react with a single sequential (linear) antigenic determinant on all three P proteins, but that additional antibodies recognize a conformational determinant(s).

Systemic lupus erythematosus (SLE) is an autoimmune disease generally characterized by serum antibodies directed against nuclear proteins and nucleic acids (1). That some patients' sera contain antibodies against ribosomal constituents (2, 3) has long been known, but the identity of these ribosomal antigens has only recently been determined (4). SLE anti-ribosome antibodies show almost exclusive reactivity against three 60S ribosomal subunit phosphoproteins called P0, P1, and P2 (4, 5). These same three proteins are also recognized by a mouse monoclonal antibody raised against chicken ribosomes (6). These "P" proteins have molecular sizes of ≈ 38 , 19, and 17 kDa, respectively. P1 and P2 are believed to be the eukaryotic equivalent of the *Escherichia coli* ribosomal protein L12 and have been shown to contain sequences that are highly conserved among eukaryotes (6). Thus, P2 (7) from rat liver shows a high degree of amino acid sequence homology with *Artemia salina* ribosomal protein eL12 (8) and yeast ribosomal protein YPA1 (9). P1 and P2 also appear to be the functional counterparts of *Artemia* ribosomal proteins eL12' and eL12 and yeast ribosomal proteins YPA1/YPA2 (10-12). In order to further evaluate the remarkable specificity of the SLE anti-P0, -P1,

and -P2 (anti-P) antibodies, we have mapped the antigenic determinant on the P proteins. This determinant is present on all three proteins and is contained within a common sequence of 22 amino acids at the carboxyl terminus of the *Artemia* ribosomal proteins eL12 and eL12'.

MATERIALS AND METHODS

Preparation of Ribosomes and Ribosomal Protein. High salt-washed ribosomes were isolated from rabbit reticulocytes and *Artemia* as previously described (13). Ribosomal protein eL12 was purified from *Artemia*, as described previously (14), with the exception that the protein was precipitated from the ethanol/ammonium chloride extract with 10% trifluoroacetic acid instead of acetone. The antiserum used was raised in rabbits (11, 14) against a mixture (4:1) of proteins eL12 and eL12'. No antibodies, however, against protein eL12' were detected in the serum. A 0.15 M saline-soluble dog spleen extract was also a source of ribosomal proteins P0, P1, and P2 (15). Sera containing antibodies against the ribosomal P proteins came from SLE patients (4, 16).

HeLa cells were grown in Joklik's minimal essential medium, recovered by centrifugation, and disrupted in a Dounce homogenizer. The homogenate was centrifuged for 20 min at $30,000 \times g$; the postmitochondrial supernatant was removed and centrifuged at $100,000 \times g$ for 5 hr. This supernatant was then removed and the pelleted ribosomes were suspended in 20 mM Tris-Cl, pH 7.4/5 mM $MgCl_2/0.5$ M KCl and recovered after centrifugation at $100,000 \times g$ for 5 hr.

$[^{35}S]$ Methionine-labeled ribosomal proteins were prepared by protein A-facilitated precipitation (17). Briefly, HeLa cells were grown in Joklik's minimal essential medium to a cell density of 5×10^5 cells/ml. The cells were centrifuged and resuspended in the described medium lacking methionine. The cells were then incubated for 16 hr in the presence of $[^{35}S]$ methionine (10 $\mu Ci/ml$; 1 Ci = 37 GBq), and ribosomes were prepared as previously described. The labeled ribosomes were then dissociated with RNase (20 $\mu g/ml$) and EDTA (10 mM) for 20 min at room temperature.

Synthesis of Peptides. Fig. 1 shows the sequences of two peptides, corresponding to the first 20 (N-terminal) and last 22 (C-terminal) amino acids of *Artemia* ribosomal protein eL12 (8). These peptides were synthesized by solid phase methods (18). Deprotection and cleavage from the resin were achieved by treatment with anhydrous HF according to the procedure of Tam *et al.* (19). The peptides were first purified by gel filtration on Sephadex G-25 and finally by preparative HPLC using a μ Bondapak C_{18} column. The purity of the

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Abbreviations: SLE, systemic lupus erythematosus; C-terminal, carboxyl-terminal of ribosomal protein eL12; N-terminal, NH₂-terminal of protein eL12; P proteins, eukaryotic ribosomal proteins P0, P1, and P2.

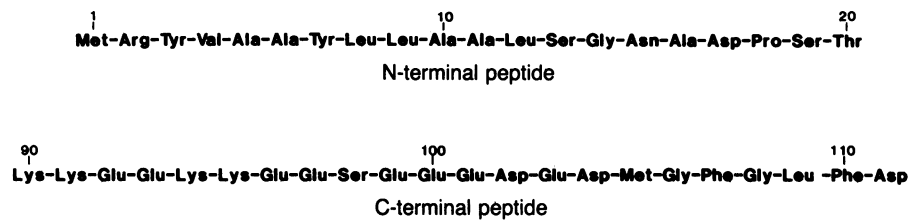


FIG. 1. Amino acid sequences of the N- and C-terminal peptides of *Artemia* ribosomal protein eL12.

peptides was confirmed by analytical HPLC, amino acid analysis, and microsequencing. The peptides were conjugated to bovine thyroglobulin as described by Chopra *et al.* (20).

Assays. Analytical isoelectric focusing was performed in 5% polyacrylamide slab gels containing 8 M urea, 2% wt/vol Pharmalyte (pH ranges 5–8 and 3–10 in a 4:1 ratio) and 2% wt/vol Nonidet P-40. Proteins were electrophoretically blotted to nitrocellulose paper in 0.7% acetic acid. Proteins were separated under nondenaturing conditions on a 10% polyacrylamide gel using 90 mM Tris/80 mM boric acid/2.5 mM EDTA, pH 8.4, as both gel and running buffers. The same buffer was also used to electrophoretically blot proteins to nitrocellulose paper. Polyacrylamide gel electrophoresis, immunological blotting, and probing of nitrocellulose transfers were done as described (15, 21–23).

Antibody reactivity with the C-terminal or N-terminal peptides coupled to thyroglobulin was tested by a dot blot assay (24). Briefly, 8 ng of peptide was applied directly to nitrocellulose paper (BA 83, Schleicher & Schuell). After blocking protein-free sites with 3% (wt/vol) bovine serum albumin, the nitrocellulose strips were sequentially probed with test antibody followed by an anti-human or anti-rabbit ¹²⁵I-labeled goat second antibody. Partial proteolysis of protein eL12 in the presence of 0.5% NaDodSO₄ (25) was accomplished with trypsin (Worthington) and staphylococcal V8 protease (Miles).

RESULTS

Extraribosomal Location and Macromolecular Structures of P0, P1, and P2. It has been reported that ribosomal protein eL12 from *Artemia* is also found in the ribosome-free cytoplasm (14). To determine whether the same situation exists in mammalian cells, HeLa cell ribosomes and a postribosomal supernatant were subjected to immunological blot analysis using SLE anti-P as a probe. Fig. 2A shows that SLE anti-P reacts with proteins P0, P1, and P2 from both the ribosome (lane a) and the cytoplasm (lane b). When the proteins were analyzed after isoelectric focusing (Fig. 2B), it was observed that the supernatant forms of P0 and P1 (lane b) were more acidic than their ribosomal counterparts (lane a). Because these proteins are known to be phosphorylated (4–6, 26, 27), the mobility change could be due to a difference in the degree of phosphorylation between the ribosomal and cytoplasmic proteins. These results show that the presence of the proteins in the supernatant was not the result of contaminating or degraded ribosomes. Zinker and Warner (28) have reported that, *in vivo*, a yeast ribosomal protein homologous to P1/P2 can exchange with its cytoplasmic counterpart.

In attempts to purify P0, P1, and P2 by a number of techniques including preparative isoelectric focusing, DEAE chromatography, and gel filtration, it was found that all three proteins migrated together. Similar results were obtained whether the proteins were isolated from ribosomes or a saline-soluble spleen extract (see *Materials and Methods*). Fig. 3A shows that P0, P1, and P2 comigrate as a single band after polyacrylamide gel electrophoresis under nondenaturing conditions but are separated when electrophoresed in a second dimension in the presence of NaDodSO₄. Chroma-

tography on a Sephadex G-200 column and immunological blot analysis of the fractions revealed that the bulk of P0, P1, and P2 elute together from the column at a molecular size of ≈140 kDa (Fig. 3B). The close similarity in function and structure between P1/P2 and the *E. coli* ribosomal protein L12 suggests that P0, P1, and P2 formed a complex similar to that observed with *E. coli* ribosomal proteins L12 and L10. In the latter case, it has been found that two dimers of L12 combine with a molecule of L10 to form a pentameric complex (29).

Epitopic Mapping—Proteolytic Digestion. Since the three P ribosomal proteins are known to have at least one common antigenic site (4, 6), it was of interest to compare the reactivity of the SLE anti-P antibodies and an antibody preparation raised against *Artemia* protein eL12. Fig. 4A shows the results of an experiment in which reticulocyte ribosomal proteins were separated by gel electrophoresis, blotted to nitrocellulose paper, and reacted with either anti-P or anti-eL12 antibodies. The anti-P antibodies reacted with P0, P1, and P2 (lane a), whereas the *Artemia* anti-eL12 (12) antibody reacts almost exclusively with its homologous reticulocyte ribosomal protein (lane b). The nature of the two minor larger molecular size bands are not known. In order to investigate the possibility that different antigenic sites were being recognized by the different antibodies, purified protein eL12 was partially digested with either staphylococcal V8 protease or trypsin, and the reaction products were analyzed by immunoblotting using anti-P and anti-eL12 antibodies. Fig. 4B (lane a) shows the reaction of the undigested protein

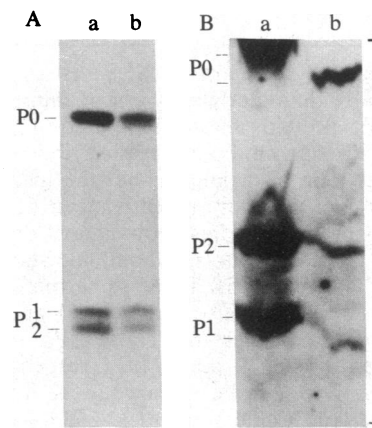


FIG. 2. (A) Immunological blot detection of the P proteins from ribosomes (lane a) and postribosomal supernatant (lane b) of HeLa cells using a SLE anti-P serum. The ribosomes and postribosomal supernatant were obtained as described in *Materials and Methods* and subjected to polyacrylamide gel electrophoresis (12%) containing 0.1% NaDodSO₄, followed by immunological blotting. (B) Isoelectric focusing of HeLa cell ribosomal proteins (lane a) and postribosomal supernatant proteins (lane b). The extracts were focused in a 5% polyacrylamide gel containing 8 M urea, blotted to nitrocellulose, and probed with the same SLE anti-P serum used in A. Protein samples were applied to the top of the gel. The P0 protein has a near neutral pI (4) and does not migrate very far into the gel. The position of the anode (+) and cathode (-) are shown.

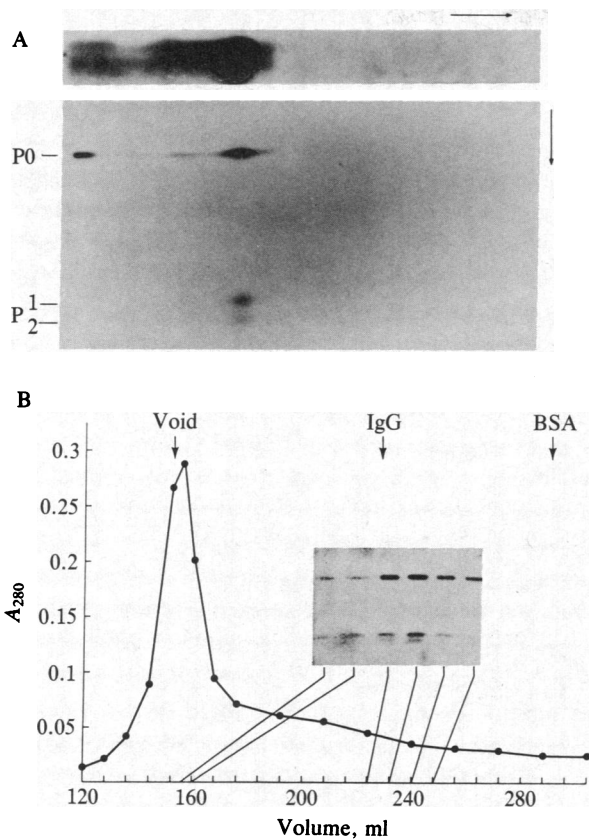


FIG. 3. (A) Two-dimensional gel electrophoretic analysis of a saline-soluble dog spleen extract. The extract was electrophoresed under nondenaturing conditions (see *Materials and Methods*) in the horizontal dimension. A gel strip was excised, equilibrated in Laemmli upper gel buffer (21) containing 0.5% NaDodSO₄, and applied to a 12% polyacrylamide gel containing 0.1% NaDodSO₄ (vertical direction). The P proteins were detected by immunoblotting using a SLE anti-P serum. (B) Sephadex G-200 chromatography of the P complex from a soluble dog spleen extract. Fifty milligrams of protein was applied to a Sephadex G-200 column (2.5 × 90 cm) equilibrated with 0.01 M phosphate buffer, pH 7.5, containing 0.15 M NaCl. The column was eluted with this same buffer at a flow rate of 12 ml/hr, and 4-ml fractions were collected. Every alternate fraction was subjected to dot blot analysis, and the positive fractions were analyzed by immunological blotting as shown in the inset above. BSA, bovine serum albumin.

eL12 with anti-eL12. Lanes b and c show that the *Artemia* anti-eL12 antibody reacts with a 9-kDa cleavage product of both the V8 protease and trypsin digests, but the SLE anti-P does not recognize these fragments (lanes d and e). The higher molecular size reactive material in the case of the trypsin digests (lanes c and e) represents undigested protein eL12. The inability of the anti-P antibody to react with any of the digestion products suggests that the antigenic determinant(s) recognized by the SLE anti-P is situated at one or both ends of the protein and that either the antigenic site(s) was proteolyzed or an antigenic peptide of small molecular size was lost from the gel. These results also demonstrate that the two antibody preparations anti-eL12 and anti-P are recognizing different epitopes.

Synthetic Peptides. Previous sequencing results have shown that *Artemia* ribosomal proteins eL12 (P2) and eL12' (P1) have 22 residues at their C termini that are identical, whereas the N-terminal sequences are dissimilar (30). In addition, the C-terminal sequence appears to be strongly conserved in other organisms (7, 30). Thus, we speculated that this C-terminal sequence might be involved in the antigenic reaction of SLE anti-P with these proteins. The

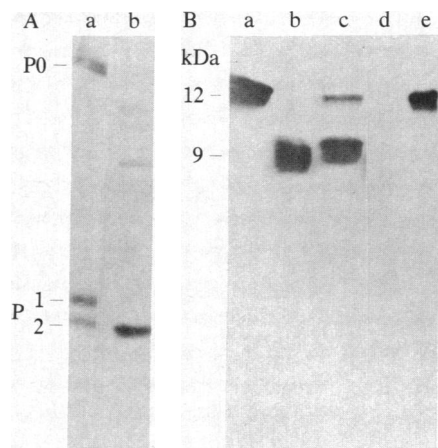


FIG. 4. (A) Immunological blot reactivity of a SLE anti-P antibody (lane a) and rabbit anti-eL12 antibody (lane b) after NaDodSO₄/polyacrylamide gel electrophoresis of *Artemia* ribosomes. (B) Immunological blot reactivity of rabbit anti-eL12 antibody (lanes a-c) and SLE anti-P antibody (lanes d and e) after partial proteolysis of purified *Artemia* protein eL12. Three micrograms of eL12 was incubated with either buffer alone (lane a), 5 µg of staphylococcal V8 protease (lanes b and d), or 2.5 µg of trypsin (lanes c and e) in the presence of 0.5% NaDodSO₄. After 30 min at 37°C the samples were boiled in 2% NaDodSO₄, and the products were resolved on a 20% polyacrylamide gel.

chemically synthesized N- and C-terminals of protein eL12 (Fig. 1) were conjugated to bovine thyroglobulin and assayed, by dot blot analysis, for their ability to react with SLE anti-P containing sera. Fig. 5 (lanes a-e) shows that five different SLE anti-P sera react with the C-terminal peptide but not with the N-terminal peptide and that serum from a normal control (lane f) fails to react with either peptide. In further studies, 16 SLE anti-P sera were tested, and all reacted exclusively with the C-terminal peptide. In contrast, 12 SLE sera that contained antibodies directed against nuclear proteins Sm and U1 RNP (ribonucleoprotein) but not against the ribosomal proteins showed no reaction against either peptide (data not shown). The rabbit anti-eL12 antibody failed to react with either terminal peptide, again showing that the two antibodies react with different epitopes.

In order to demonstrate that the same C-terminal peptide was also being recognized by the SLE anti-P in the intact proteins, the SLE anti-P antibodies were incubated with each peptide prior to being assayed by immunological blot analysis to detect the ribosomal proteins. Fig. 6 (lane a) shows the reaction of the SLE anti-P with proteins P0, P1, and P2 in the absence of the peptides. Lane b shows that the reaction of the anti-P antibodies with all three proteins is completely blocked when the antibodies are first preincubated with the C-terminal peptide. On the other hand, as seen in lane c, the addition of the N-terminal peptide is without effect.

In contrast to these results, the C-terminal peptide produced only a slight reduction in the reactivity of the SLE

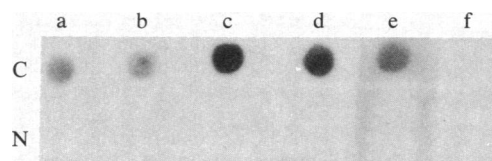


FIG. 5. Reactivity of SLE anti-P serum with the C-terminal peptide of protein eL12. Eight nanograms of either the C- or N-terminal peptide coupled to bovine thyroglobulin was applied directly to nitrocellulose paper, and the strips were probed with five different SLE anti-P sera (lanes a-e) or a normal serum (lane f).

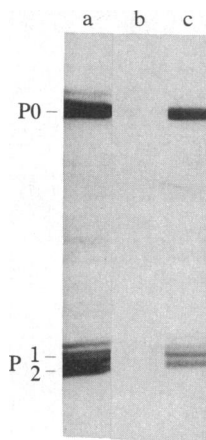


FIG. 6. SLE anti-P reactivity after incubation with the C-terminal peptide. Rabbit reticulocyte ribosomal proteins (lanes a–c) were resolved by NaDodSO₄/polyacrylamide gel electrophoresis and blotted to nitrocellulose paper. Before probing, the SLE anti-P serum was diluted 1:1000 and was incubated with buffer (lane a), 7 μ g of thyroglobulin-bound C-terminal peptide (lane b), or 7 μ g of thyroglobulin-bound N-terminal peptide (lane c).

anti-P serum with labeled HeLa cell ribosomes under *nondenaturing* conditions. Fig. 7 (lane a) shows that when SLE anti-P is incubated with [³⁵S]methionine-labeled HeLa cell ribosomes, ribosomal proteins P0, P1, and P2 can be immunoprecipitated. However, in contrast to the results observed using immunological blot analysis, preincubation of the antibody with the C-terminal peptide (Fig. 7, lane b) has only a small effect on this immunoprecipitation. The N-terminal peptide is also without effect (Fig. 7, lane c). Similarly, the reactivity of the SLE anti-P with the proteins that had been both electrophoresed and transferred to nitrocellulose under *nondenaturing* conditions was not significantly blocked by preincubation with the C-terminal peptide (data not shown). We also have observed that anti-P Fab fragments will inhibit protein synthesis in a reticulocyte *in vitro* system and that the C-terminal peptide again has only a small effect in reversing this inhibition (data not shown). All of these observations suggest that SLE anti-P containing sera contain distinct antibodies that react with either linear or conformational determinants located on the P proteins.

DISCUSSION

The prokaryotic equivalent of the eukaryotic ribosomal proteins P1 and P2 is presumed to be *E. coli* ribosomal protein L12. There are many similarities in structure and function between these proteins. Thus, they both are found on the ribosome in multiple copies (14, 31), exist in solution as dimers (14, 32–36), and are required for the functional activity of the ribosome (10–12, 37). In addition, *E. coli* ribosomal protein L10 is required for the binding of L12 to the ribosome (38, 39), and it has been shown that in solution L12 forms a pentameric complex with L10 in the ratio of 4:1 (29), comparable to that found on the ribosome (31, 40). Although a protein comparable to L10 has not previously been de-

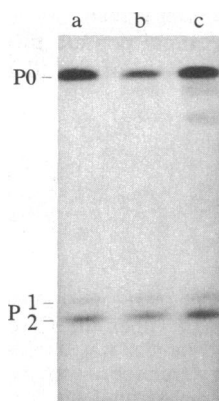


FIG. 7. Immunoprecipitation of [³⁵S]-methionine-labeled P proteins from HeLa cell ribosomes. [³⁵S]Methionine-labeled HeLa cell ribosomal proteins were prepared as described in *Materials and Methods*. Eighty micrograms of total IgG from an anti-P serum bound to Sepharose-protein A was preincubated for 60 min at 23°C with buffer (lane a), 20 μ g of thyroglobulin-bound C-terminal peptide (lane b), or 20 μ g of thyroglobulin-bound N-terminal peptide (lane c) prior to incubation (30 min, 4°C) with the labeled ribosomal proteins. The immune complex was centrifuged, washed, eluted with Laemmli sample buffer (21), and the proteins were resolved by NaDodSO₄ gel electrophoresis.

scribed in eukaryotes, the copurification of P0, P1, and P2 suggests that these three proteins are tightly complexed and that P0 may be the eukaryotic equivalent of L10. This is supported by the observation that all three proteins migrate together when electrophoresed under *nondenaturing* conditions and that when a ribosomal extract was chromatographed on Sephadex G-200, all three proteins emerged together at a molecular size of approximately 140 kDa.

All three P proteins were found in the ribosome-free cytoplasm of HeLa cells. It is noteworthy that the P0 and P1 of the cytoplasm are more acidic than their ribosomal counterparts, perhaps due to the degree of phosphorylation of these proteins (4, 6). The functional significance of this cytoplasmic pool is not known. A large cytoplasmic pool of the homologous proteins has also been found in *Artemia* (14), although in that study no attempt was made to investigate whether the cytoplasmic proteins differed from the ribosomal proteins.

The antigenic determinant recognized by the SLE anti-P autoantibodies was thought to reside in one or both ends of protein eL12, because limited proteolytic digestion with trypsin or V8 protease caused only a small change in the apparent molecular size of the protein but resulted in a total loss of antibody reactivity. The antibody against *Artemia* protein eL12, on the other hand, reacted with a cleavage product from both digestions, clearly demonstrating that the two antibody preparations recognized different epitopes. Previous results have indicated that P0, P1, and P2 contain at least one common epitope (4–6), and it has also been determined that *Artemia* proteins eL12 and eL12' have an identical 22-amino acid C-terminal sequence (30). In addition, considerable homology between the carboxyl, but not the amino, terminus of the equivalent proteins in other eukaryotes has been observed (7, 30). Since the SLE anti-P antibodies recognized both proteins eL12 and eL12' as well as their equivalents in all eukaryotes tested (4, 5), the antigenic determinant was predicted to lie within the 22 amino acids of the C terminus. Dot blot analysis of the synthetic C- and N-terminal peptides showed that the anti-P antibodies reacted only with the C-terminal peptide. The ability of the C-terminal peptide to completely inhibit the reactivity of the anti-P antibodies with all three proteins on an immunological blot proved that the SLE autoantibodies recognized the same sequential determinant on P0, P1, and P2. In addition, other experiments have shown that there is no precipitating activity by double diffusion in agarose suggesting that only a single epitope is present within the 22 C-terminal amino acids (data not shown). Although the sequence of P0 has not been determined, it would be predicted to contain all or part of the C-terminal peptide used in these studies.

Antigenic sites on histones (41) and the La protein (42) in SLE have already been inferred from proteolytic cleavage data or the hydrophilicity index of predicted amino acid sequences, respectively, but the present study positively identifies a linear or "sequential" (43) antigenic peptide on an autoantigen in SLE. Identification of the antigenic determinant and the ability to synthesize the peptide antigen have important implications. The specificity of lupus autoantibodies for 3 proteins out of a total of approximately 80 ribosomal proteins (4, 5) is now shown to be further restricted to a single sequential determinant present on all 3 proteins. Since globular proteins contain, on average, one epitope per 5 kDa (44), anti-P restriction to a determinant within 22 amino acids (about 2.4 kDa) is compatible with theories of autoantibody production, such as antibody stimulation by cross-reaction with a foreign antigen (bacterial or viral), or random activation of a T helper or B lymphocyte clone (1). In these cases, antibody reactivity with the three P proteins would be a chance phenomenon related to a shared sequence on the

foreign protein or due to the limited specificity of the B- or T-cell clone activated. Using a computer program (National Biomedical Research Foundation data bank), we have not found striking homologies between the C-terminal peptide and other nonribosomal protein sequences.

In addition to the identification of a sequential determinant, evidence for antibodies recognizing conformation(s) of the P proteins was obtained. Although the C-terminal peptide completely blocked reactivity with the P proteins on an immunological blot when the proteins were denatured by NaDodSO₄, the peptide did not block anti-P reactivity with the *non-denatured* proteins on nitrocellulose paper and did not significantly inhibit immunoprecipitation of [³⁵S]methionine-labeled P proteins. Furthermore, the synthetic peptide could only modestly reverse antibody-mediated inhibition of *in vitro* protein synthesis (data not shown). All of these findings indicate that SLE anti-P sera contain antibodies to both conformational and sequential determinants. This complexity of antibody production is similar to that seen following experimental immunization where antibodies specific for the conformation of the native protein are dominant (44). These findings are also similar to other studies with synthetic peptides where polyclonal rabbit antisera raised against an intact influenza hemagglutinin (HA-1) failed to react with 20 synthetic peptides corresponding to 75% of the HA-1 molecule (45). Overall, the results obtained in this study, together with the evidence for sera from different patients reacting with different proteins from the same ribonucleoprotein particle (23) or different epitopes on the same protein (46, 47) suggest a coordinate polyclonal immune response to selected proteins in SLE and related diseases.

Most of the protein autoantigens of clinical interest in SLE are intracellular (1) and therefore difficult to purify to homogeneity. The synthesis of an antigenic peptide may represent an important advance, especially for diagnostic testing. Immunoassay of SLE anti-P antibodies using the synthetic peptide appears to be superior to all other methods of detection in terms of rapidity, sensitivity, specificity, and ability to quantitate antibody levels.

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