Small nuclear RNA-associated proteins are immunologically related as revealed by mapping of autoimmune reactive B-cell epitopes

(autoepitopes/epitope mapping/common domains/ribonucleoproteins/peptide ELISA)

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Communicated by Joan A. STEITZ, March 20, 1989

ABSTRACT Autoantibodies from a patient with systemic lupus erythematosus, which recognize U1 and U2 small nuclear ribonucleoprotein particles (snRNPs), were used to map B-cell autoepitopes on the U1 snRNP-specific A protein. This protein contains two regions that are highly similar to regions in the U2 snRNP-specific B' protein. A site termed epitope 2 maps in one such region and was found to react with antibodies cross-reactive between A and B'. A second site, epitope 1, is situated in a proline-rich region that shows no homology with B'. This epitope can bind three different autoantibodies with distinct specificities. Epitope 1-affinity-purified antibodies from different patients react with either (i) the A protein exclusively; (ii) proteins A, B'/B, a synthetic peptide for part of the N polypeptide, and an unidentified protein with a molecular mass of 50 kDa; or (iii) proteins A, B', C, and the N-derived peptide. Comparison of the primary structures of proteins B'/B, N, and C reveals multiple epitope 1-like sequences in all of them. The possibility that these repeating regions act as immunogens in patients with autoimmune diseases is discussed.

The U small nuclear RNA (snRNA)-containing small nuclear ribonucleoprotein particles (snRNPs) play key roles in the maturation process of pre-mRNAs (1). The five most abundant nucleoplasmic U snRNAs are organized in four different snRNPs: U1, U2, U4/U6, and U5 (U1–U6) snRNPs. All four snRNPs particles contain the so-called Sm domain, consisting of proteins called B', B, D, D', E, F, and G. In addition to this core, U1 snRNPs contain three specific polypeptides, A, C, and the 70-kDa peptide, whereas U2 snRNPs are characterized by the presence of two unique proteins, A' and B'' (2). Recently another snRNP-associated polypeptide designated "N" was identified, which is expressed predominantly in brain (3).

Autoantibodies against snRNPs often spontaneously occur in sera from patients with connective tissue diseases (reviewed in ref. 4). The primary event that triggers autoantibody production is not known, but there is increasing evidence that this reaction might be antigen-driven (4, 5).

Recently we described the molecular cloning of a cDNA containing the entire coding sequence for the U1 snRNP-specific A protein (6). This protein presents an excellent model for studies of autoimmune phenomena in systemic lupus erythematosus (SLE) and related diseases because at least three different autoantibody responses against this protein have been described. Anti-A antibodies occur in (i) anti-(U1)RNP sera commonly obtained from patients with mixed connective tissue disease (7, 8); (ii) in anti-Sm [anti-(U1–U6)RNP] sera (6, 9), almost exclusively obtained from patients with SLE; and (iii) in anti-(U1, U2)RNP sera (10), which do not show a strong correlation with disease. Information on the exact structure of B-cell epitopes recognized by antibodies in these three classes of sera might provide insight into the nature of the autoimmune abnormalities.

We describe here three different populations of autoantibodies that cross-react with proline-rich regions in the snRNP proteins A, B'/B, N, and C and with an as-yet-undefined 50-kDa protein. Our results support the hypothesis that autoantibody production is antigen-driven and show that the immune response against individual snRNP proteins can differ from patient to patient.

MATERIALS AND METHODS

Construction and Screening of a HA4-DNase Fragment Library. One microgram of the EcoRI cDNA insert of the previously described clone HA4-4 (6) was digested with 2 ng of DNase I in 10 μl of 20 mM Tris (pH 7.4) in the presence of 1.5 mM Mn²⁺ at room temperature (11). Aliquots (3 μl) were taken at 3, 4, and 5 min and pooled. DNA was made blunt-ended with T4 polymerase, dialyzed against H₂O, and ligated to phosphorylated EcoRI linkers (12). The material was digested with EcoRI and fractionated on 8% acrylamide gels to remove unligated linkers. Fragments of 50–250 base pairs (bp) were eluted, and 200 ng of the DNA was ligated into λgt11 arms and packaged into phage heads (Promega Biotec). Recombinant phages were amplified in Escherichia coli Y1090 (13) and plated at a density of 3 × 10⁴ per 150-cm² plate. Screening with the previously described (10) human autoimmune serum V26 was performed as described (14).

Characterization of Autoimmune Sera. The autoimmune sera used in this study were analyzed by immunoblotting with nuclear extract from HeLa cells (15), by counter immunoelectrophoresis (16), and by immunoprecipitation from radiolabeled cell extracts (10). Characterization of sera was done as follows. Sera that precipitated exclusively U1 snRNPs were in all cases found to recognize one or more of the U1 snRNP-specific proteins (A, C, or the 70-kDa protein) and were classified as anti-(U1)RNP. All sera containing antibodies reactive with U1 and U2 snRNPs were found to recognize B'' and A and sometimes also one of the U1 snRNP-specific 70-kDa or C proteins. These sera were classified as anti-(U1, U2)RNP. Sera precipitating U1, U2, U5, and U4/U6 were classified as anti-Sm. Sometimes sera contained both anti-Sm and anti-(U1)RNP antibodies. This was the case when disproportionate amounts of U1 snRNPs, as compared with U2 snRNPs, were precipitated. These sera are referred to as anti-RNP/Sm.

Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle.
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### Analysis of Recombinant Antigen and Affinity Purification of Antibody

Expression of recombinant antigen was induced with isopropyl β-D-thiogalactoside as described (13, 14). Bacterial lysates (14) were fractionated on preparative 10% acrylamide gels and blotted onto nitrocellulose. Strips containing the recombinant β-galactosidase fusion protein were cut out and used for affinity purification of autoantibodies as described by Smith and Fisher (17). As a control, antibodies were eluted from blot strips containing β-galactosidase. These strips were cut from blots containing a bacterial lysate from induced E. coli strain BNN 97 (13).

**ELISA with Synthetic Peptides.** Synthetic peptides were designed from the predicted amino acid sequences of proteins A (6) and N (18) and obtained from the European Veterinary Laboratory, Biotech Division (Amsterdam) (A peptide) and from the Protein and Nucleic Acid Chemistry Facility (Yale University) (N peptides). Nunc 96-well microtiter plates were coated overnight at 4°C with these peptides at 0.5 μg per well in coating buffer (15 mM Na2CO3/35 mM NaHCO3, pH 9.6). Plates were washed five times with PBST (20 mM sodium phosphate, pH 7.3/130 mM NaCl/0.05% Tween 20), and the remaining binding sites were blocked with 1% bovine serum albumin in PBST for 2 hr at room temperature. Affinity-purified antibodies eluted from 200 μg of nitrocellulose-bound recombinant antigen (see above) were recovered in a volume of 2 ml. Fifty microliters was added to each well and incubated for 2 hr at room temperature. Bound antibodies were washed with PBST and detected as described (19).

### RESULTS

**Construction and Screening of an Epitope Library.** In a previous report, we described the isolation and characterization of a cDNA clone, λHA-4, that encodes the human U1 snRNP-specific A protein (6). This protein expresses multiple epitopes that are recognized by autoantibodies in sera from patients with certain connective tissue diseases (6, 19).

Epitope-containing sequences within the A protein could be precisely mapped by screening a sublibrary made in Agt11 that contained 50- to 250-bp-long random DNA fragments from the 1.2-kilobase EcoRI insert of clone λHA-4. Screening was performed with the human anti-(U1,U2)RNP serum V26 (10).

From a total of 2 x 10^6 individual phages, 18 were found that encoded fusion proteins reactive with antibodies in the V26 serum. Four appeared to contain more than one insert and were excluded from further study. The inserts from the remaining 14 were subcloned into phage M13, and their sequences were determined (20). All clones were found to be in the correct reading frames to allow expression of fusion proteins containing β-galactosidase and parts of A.

**Definition of Two Autoepitopes on the A Protein.** All 14 clones from which immunoreactive fusion proteins could be derived were found to encode amino acid sequences situated in the C-terminal half of the coding region of the A protein. When the amino acid sequence of an epitope is designed as the minimum predicted sequence shared by clones with similar immunologically reactive properties, two separate epitopes can be identified (Fig. 1). Epitope 1 covers amino acids 165–185 and is situated in a sequence unique to the A protein (nonshaded area in Fig. 1), whereas epitope 2 (amino acids 232–256) lies in a region of the A protein that shows extensive homology with a region of the B′′ protein (shaded area in Fig. 1; refs. 6 and 14).

Evidence that these epitopes are distinct entities was obtained from experiments that show that different antibody populations bind to epitopes 1 and 2. A Agt11 clone encoding amino acids 165–190 of the A protein (hereafter termed clone A165–190) was induced, and the expressed β-galactosidase fusion protein was used to affinity-purify autoantibodies from serum V26. The affinity-purified antibodies reacted exclusively with the A protein when they were used to probe a blot containing total nuclear proteins derived from HeLa cells (Fig. 2, lane ep1). Antibodies affinity-purified from serum V26 by using epitope 2 (as contained in a clone termed A232-268), on the other hand, recognized a cross-reactive epitope on A and B′′ (Fig. 2, lane ep2).

As a next step we wished to establish whether the recognition of epitope 2 was a specific feature of antibodies in serum V26 or a more general phenomenon. Therefore, we used a panel of 26 anti-snRNP sera composed of anti-

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**Fig. 1.** Epitope mapping of the U1 snRNP-specific A protein. A DNase I fragment library of the full-length clone λHA-4 (6) was screened with the anti-(U1,U2)RNP serum V26. Fourteen immunoreactive clones were isolated, and their DNA sequences were determined. Numbers at the right refer to amino acids encoded by the respective clones. The minimal overlapping sequences necessary for immunoreactivity are indicated as epitopes. Shaded areas indicate regions where the A protein has extensive homology with B′′ (6, 14).

**Fig. 2.** Affinity purification of epitope 1- and epitope 2-reactive antibodies. Blots containing nuclear proteins from HeLa cells were probed with serum V26 (lane Σ) or antibodies affinity-purified from V26 by using epitope 1- or epitope 2-containing β-galactosidase fusion proteins (lanes ep1 and ep2, respectively). Bands additional to A and B′′ in lane ep2 are always observed with anti-B′′ sera (10, 14) and are considered to be degradation products of B′′. 70K, U1 snRNA-associated 70-kDa protein.
Fig. 3. Epitope 1-reactive Sm antibodies. (A) Immunoblots containing nuclear proteins from HeLa cells (nuclei) or anti-mG affinity-purified snRNPs (21) were probed with serum K22 (lane $\Sigma$) or antibodies affinity-purified from K22 by using either a full-length $\beta$-galactosidase A fusion protein (lanes tot.A; ref. 5) or an epitope 1-containing fusion protein (lanes epip1). 70K and 50K, 70- and 50-kDa. (B) RNA analysis of $^{32}$P-labeled cell extracts (lane RNA) immunoprecipitated with serum K22 (lane $\Sigma$) or affinity-purified fractions as in A.

(U1)RNP (n = 9), anti-Sm (n = 9), and anti-(U1,U2)RNP sera (n = 8). None of the anti-(U1)RNP or anti-Sm sera recognized epitope 2 as defined by their lack of reactivity with fusion proteins encoded by clones A153-190 and A158-185. None of the nine anti-(U1)RNP sera reacted with either of the clones, whereas six of nine anti-Sm sera recognized epitope 1. Four of eight anti-(U1,U2)RNP sera recognized epitope 2 expressed by both clones. Therefore, we conclude that epitope 2 is exclusively recognized by A and B$''$ cross-reactive antibodies in some anti-(U1,U2)RNP sera.

Epitope 1-Like Sequences Are Present in Other snRNP Proteins. Epitope 1 was subjected to a more detailed study. The same panel of anti-snRNP sera used previously was tested on immunoblots containing fusion proteins encoded by clones A153-190 and A158-185. None of the nine anti-(U1)RNP sera reacted with either of the clones, whereas six of nine anti-Sm sera recognized epitope 1. Four of eight anti-(U1,U2)RNP sera also reacted with epitope 1, two of which (including serum V26) were already known to react with epitope 2 as well (see above). So epitope 1 bound antibodies from some anti-Sm sera as well as from some anti-(U1,U2)RNP sera.

When the epitope 1-binding antibodies from an anti-Sm serum called K22 were eluted from blots containing fusion proteins encoded by either A158-185 or A165-190 and used to reprobe blots with nuclear proteins from HeLa cells, these antibodies behaved differently from the anti-epitope 1 antibodies in serum V26. Instead of a reaction with the A protein (as was the case for V26-derived antibodies, Fig. 2) anti-epitope 1 antibodies from serum K22 predominantly reacted with proteins B$'$/B and an as-yet-unidentified protein with a molecular mass of 50 kDa (marked as 50K in Fig. 3A). Almost no reaction with the A protein was detectable, suggesting a much higher affinity of these antibodies for the B$'$/B and 50-kDa proteins. Incubation of the same antibodies with blots containing snRNP proteins showed that the 50-kDa protein was not an integral part of snRNP, whereas A and B$'$/B were recognized in approximately the same ratio as observed in total nuclear protein blots (Fig. 3A). That these antibodies still recognized Sm is shown in Fig. 3B. Epitope 1 affinity-purified antibodies from serum K22 precipitated U1-U6 snRNPs from a radiolabeled HeLa cell extract. It is interesting to note that serum K22 also recognized additional epitopes on the A protein. When antibodies from K22 were bound to and then eluted from full-length recombinant A protein, in addition to recovering anti-epitope 1 antibodies, antibodies strongly reactive with the A protein were recovered as well (Fig. 3A, lane tot.A).

The simplest explanation for the observed cross-reactivities is that the A, B$'$/B, and 50-kDa proteins have common epitopes. Therefore, epitope 1 was compared with the primary sequence of B$'$/B and an earlier described B-like, 24,614-Da snRNP protein called "N,$" which is found predominantly in brain (3, 18). In the carboxyl terminus of N, six versions of epitope 1 could be detected with a similarity ranging from 47% to 63% (Fig. 4). Since the primary sequence of N is highly homologous to B$'$/B (J. Hardin, Yale University, personal communication), several epitope 1 versions are also present in the B proteins, meaning that this is the region that most likely binds the epitope 1-purified antibodies. Because proteins N and B$'$/B can be difficult to resolve on immunoblots from sodium dodecyl sulfate/polyacrylamide gels (3, 18), a different assay was used to substantiate the hypothesis that epitope 1-eluted antibodies react with protein N. We developed an ELISA with synthetic epitope 1 (peptide A) and three peptides designed from N (sequences shown in Fig. 4). Fig. 5 shows that epitope 1 antibodies purified from

![Alignment of epitope 1 and protein N. The deduced amino acid sequence of N (ref. 18) was compared with that of epitope 1 by using the LFASTA computer program (22). Similarities >45% in at least 19 amino acids are shown. N1 and N2 peptides were designed from a region with the highest similarity. a.a., Amino acids.](image-url)
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FIG. 6. Epitope 1-eluted antibodies crossreact with the U1 snRNP-specific C protein. Blots containing HeLa cell nuclear proteins were probed with sera C03 or H13 (lanes 2) or antibodies affinity-purified from these sera by using either full-length β-galactosidase C fusion protein (lanes C-F.P.; ref. 23) or epitope 1-containing fusion protein (lanes epil). Asterisks indicate background binding of the peroxidase-labeled second antibody. This is prominent in the strips incubated with affinity-purified antibodies, most probably because these strips require an incubation time up to 6 times longer with the peroxidase substrate. Only this band was present in the control blot strips incubated with antibodies affinity-purified with β-galactosidase (not shown). 70K, 70 kDa.

FIG. 7. ELISA with epitope 1-affinity-purified antibodies from sera C03 or H13. For details see the legend of Fig. 5.

DISCUSSION

We previously identified a cDNA clone, called AHA-4, encoding the human U1 snRNP-associated A protein (6). In this study we have used a DNase I sublibrary (a so-called epitope library; ref. 11) of the original cDNA clone to define two regions on the A protein that interact with autoantibodies from anti-(U1, U2)RNP and anti-Sm sera.

The region identified in this study as epitope 2 on protein A differs only in two amino acids from a corresponding region (amino acids 175–199) in protein B'" (6, 14). Therefore, it was not surprising that this site was recognized by A and B'" cross-reactive antibodies. However, this site cannot be the only cross-reactive epitope because four other anti-U1, U2RNP sera did not react with epitope 2, whereas they recognized full-length B'" when expressed as β-galactosidase fusion protein (W.J.H., unpublished data). Such a polyclonal antibody response was also found in the case of proteins A and C (see below).

Epitope 1 was particularly interesting because it was recognized by three different populations of autoantibodies (Table 1). The first population was found in the anti-(U1, U2)RNP serum V26 and reacted with a sequence within epitope 1 that is not present in any of the other snRNP proteins. The second antibody specificity was present in the anti-Sm serum K22 and also recognized a sequence within epitope 1 but reacted more strongly with proteins B'/B and an unidentified 50-kDa protein as well as a peptide representing part of N than they did with A. This 50-kDa protein is not the La protein, as the reactive antibodies did not recognize cloned or natural La protein on immunoblots (W.J.H., unpublished data). Also it is probably not a snRNP protein because it is lost upon purification of snRNPs from cell extracts with anti-m7G antibodies (Fig. 3A). A third antibody specificity that could be affinity-purified from epitope 1 reacted with proteins A, B'/B, and C and with a peptide representing part of N.

It was surprising that the epitope 1-affinity-purified antibodies derived from sera K22, C03, and H13 reacted only weakly with the A protein, whereas they reacted strongly with proteins B'/B (Figs. 3A and 6). This could mean that the epitope 1 sequence is hidden from the immune system in the intact A protein. However, anti-epitope 1 antibodies derived from serum V26 react well with the A protein. Therefore, we favor the alternative explanation that epitope 1-reactive antibodies in sera K22, C03, and H13 have a higher affinity for unmodified full-length protein.

FIG. 8. Alignment of epitope 1 and protein C. For details see the legend of Fig. 4. Similarities > 35% in at least 18 amino acids are shown.
for proteins B'/B than they have for A. This is supported by the finding that these antibodies react more strongly with peptide N1 than they do with peptide A (Figs. 5 and 7).

We have further shown that the immune reaction against snRNP proteins B", A, and C is polyclonal. When the antibody populations purified by using full-length A and C proteins (Fig. 3A, lane tot.A, and Fig. 6, lanes C-F.P.) are compared with those purified by epitope 1, it is clear that the full-length proteins bound additional antibodies that reacted strongly with A and C, respectively. So proteins A and C bind both monospecific and cross-reactive antibodies. The finding that the anti-snRNP immune response is polyclonal together with the observed differences in affinity of the epitope 1 reactive antibodies supports the intriguing hypothesis of Reuter and Lührmann (5) that anti-snRNP antibodies can be elicited against endogenous snRNP particles. The repetitive proline-rich regions described here are apparently a prominent target for the immune system in many patients with autoimmune diseases.

Which protein(s) in snRNPs trigger the autoimmune response? Probably more than one. For example, anti-epitope 1 antibodies, like the ones present in serum V26, are in our view most likely elicited against the endogenous A protein. Meanwhile, anti-epitope 1 antibodies in serum K22 are probably initially elicited against either the B'/B, N, or 50-kDa proteins. Recent experiments have shown that such a supposition could be true. Antibodies with Sm specificity crossreactive with proteins A, B'/B, and N were raised upon immunization of rabbits with peptide N1 (3, 18), whereas immunization with a synthetic peptide comprising epitope 1 (peptide A in Fig. 5) resulted in anti-(U1)RNP antibodies exclusively reactive with the A protein (W.J.H., unpublished data).

Neither epitope 1 nor epitope 2 reacted with anti-A antibodies from patients' anti-(U1)RNP sera. A limitation of most epitope mapping strategies (and that is also true for the method used here) is the difficulty in detecting discontinuous or conformational epitopes in which the reactive sites are far apart in the primary structure of the antigen. Deletion mutagenesis studies show that most anti-(U1)RNP sera recognize a nonlinear epitope on the A protein (W.J.H., unpublished data). The two parts of this epitope are >125 amino acids apart, so they could not be detected in the sublibrary used in this study.

The presence of autoantibodies in the sera of patients with systemic lupus erythematosus that are cross-reactive with several snRNP proteins seems to be a general phenomenon. Our data show that at least five snRNP proteins—A, B", B', B, C, and N—are targets of cross-reactive antibody populations. This has implications for the use of autoimmune sera as probes to study the functions of individual snRNP proteins. For instance, sera that seem (almost) monospecific for the A protein on immunoblots might contain cross-reactive antibodies against C or other proteins and vice versa. Therefore, it is difficult to attribute the observed effects in assays other than immunoblotting to the reactivity of a specific autoantibody unless one has established the affinity of the autoantibodies for potentially cross-reactive proteins in that particular system.

We are grateful to Dr. J. Hardin (Yale University) for communicating his results prior to publication. Further, we thank B. Hageman and B. de Jong from the Nijmegen laboratory for excellent technical assistance. The help of Dr. J. Leunissen with computer analysis is also gratefully acknowledged. Anti-mG antibodies were a generous gift from Dr. R. Lührmann (University of Marburg, F.R.G.). This work was supported in part by The Netherlands League against Rheumatism and by Organon Teknika. W.J.H. is a recipient of a postdoctoral fellowship from the Netherlands Royal Academy of Arts and Sciences.