Purification and Partial Sequencing of the Nuclear Autoantigen RA33 Shows That It Is Indistinguishable from the A2 Protein of the Heterogeneous Nuclear Ribonucleoprotein Complex

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Abstract

RA33 is a nuclear autoantigen with an apparent molecular mass of 33 kD. Autoantibodies against RA33 are found in about 30% of sera from RA patients, but only occasionally in sera from patients with other connective tissue diseases. To characterize RA33, the antigen was purified from HeLa cell nuclear extracts to more than 90% homogeneity by affinity chromatography on heparin-Sepharose and by chromatofocusing. Sequence analysis of five tryptic peptides revealed that their sequences matched corresponding sequences of the A2 protein of the heterogeneous nuclear ribonucleoprotein (hnRNP) complex. Furthermore, RA33 was shown to be present in the 40S hnRNP complex and to behave indistinguishably from A2 in binding to single stranded DNA. In summary, these data strongly indicate that RA33 and A2 are the same protein, and thus identify on a molecular level a new autoantigen. (J. Clin. Invest. 1992. 90:1061–1066.) Key words: rheumatoid arthritis • autoimmune diagnostics • antinuclear antibodies • RNA binding protein • mRNA processing

Introduction

RA is the most common autoimmune disease and affects 1–2% of the population. Despite many years of intensive research, the etiology of the disease is still poorly understood, and no definite cure is known (1). Sera from patients with RA are primarily characterized by the presence of rheumatoid factor, an autoantibody directed against the Fc portion of IgG (2). In addition, autoantibodies against components of the cell nucleus (antinuclear antibodies) and against EBV-related structures are frequently observed (3–5). However, neither rheumatoid factor nor any of the other autoantibodies have been found to be specific for RA. Recently, autoantibodies against a nuclear protein with an apparent molecular mass of 33 kD have been described (6). These autoantibodies were detected in 35% of RA patients but apart from a few exceptions not in sera from patients suffering from other autoimmune or degenerative rheumatic disorders. Therefore, the antigen was termed RA33; this was the first description of a nuclear antigen apparently specific for RA.

Disease specific immune responses to nuclear antigens have been found for various autoimmune diseases: anti-Sm and anti-dsDNA in systemic lupus erythematosus (SLE), anti-topoisomerase (ScI70) in scleroderma, or anti-trNA synthetase in dermatopolymyositis are just a few examples. Other autoantibodies, such as anti-U1 small nuclear ribonucleoprotein (snRNP), anti-Ro/SS-A, anti-La/SS-B, or antihistone, are less specific but nevertheless, they do form valuable tools for the diagnosis of connective tissue diseases (for a review, see 7). In general, the target antigens are molecules conserved in evolution, many of which have the capacity to bind DNA or RNA, such as histones, topoisomerase, or snRNP particles involved in posttranscriptional processing of pre-mRNA (8).

In contrast to snRNPs (9, 10), components of the heterogeneous nuclear ribonucleoprotein (hnRNP) complex have only rarely been described as target structures for human autoantibodies (11–13). So far, only the A1 protein has been identified as autoantigen in a variety of autoimmune diseases (14, 15). HnRNPs are large nuclear structures composed of heterogeneous nuclear RNA and ~30 different proteins with molecular masses between 34 and 120 kD (16). Six of these proteins (A1, A2, B1, B2, C1, C2) have been referred to as the hnRNP “core” proteins (17), and the sequences of their cDNAs have been recently elucidated (18–22). They belong to a group of nucleic acid binding proteins that contain an evolutionarily highly conserved RNA binding domain of ~90 amino acids, the so-called consensus sequence-type RNA binding domain (CS-RBD [23]). Little is known about the detailed structure and function of the hnRNP complex, but there is some evidence for a functional relationship to the snRNPs (24).

In this report, we describe the purification and partial sequencing of RA33. Furthermore, it is demonstrated that RA33 is present in the 40S hnRNP complex, and that it is indistinguishable from the A2 protein in affinity chromatography on single stranded DNA (ssDNA). Taken together, the data strongly suggest that RA33 and A2 are identical.

Methods

Protein purification. HeLa S3 cells or nuclei were obtained frozen from the Computer Cell Culture Center, (University of Mons, Belgium).


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1. Abbreviations used in this paper: CS-RBD, consensus sequence-type RNA binding domain; hnRNP, heterogeneous nuclear ribonucleoprotein; MCTD, mixed connective tissue disease; snRNP, small nuclear ribonucleoprotein; ssDNA, single-stranded DNA.

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Isolation of nuclei and preparation of nuclear extracts was performed as described (6). Briefly, 10 g of nuclei were incubated for 30 min in 4 ml hypotonic buffer (10 mM Hepes-KOH, 2.5 mM MgCl₂, 25% glycerol, pH 7.9) and subsequently extracted for 40 min by addition of an equal volume of the same buffer containing 0.88 M NH₄Cl. The slurry was centrifuged for 20 min at 20,000 g, and the NH₄Cl concentration of the supernatant was adjusted to 0.3 M by diluting with 10 mM Hepes-KOH, pH 7.9.

40 ml nuclear extract (~200 mg protein) was applied to a 2.5 × 20 cm heparin-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A (20 mM Hepes-NaOH, 0.3 M NaCl, pH 7.9). The column was washed with 3 vol of buffer A, and bound protein was eluted with 3 vol of buffer B (20 mM Hepes-NaOH, 1 M NaCl, pH 7.9) followed by 3 vol of buffer C (20 mM Hepes-NaOH, 1 M NaCl, 6 M urea, pH 7.9). Protein concentration of each fraction (10 ml) was measured in a microplate reader at 620 nm using the Coomassie Protein Assay Reagent from Pierce Chemical Co. (Rockford, IL). Protein-rich fractions were analyzed for the presence of RA33 by immunoblotting, as described below. RA33 containing fractions were pooled (10 mg total protein), precipitated with 0.4 g/ml ammonium sulphate, and redisolved in chromatofocusing starting buffer (20 mM Tris-CH₃COOH, 3 M urea, pH 8.7). After dialyzing against the same buffer, proteins were further separated by chromatofocusing on a 15 × 0.6 cm PBE 94 ion exchanger column (Pharmacia). A 1:8 diluted Polybuffer 96-acetic acid, pH 6, containing 3 M urea, was used as eluent (flow rate 8 ml/h, fraction size 2 ml). Aliquots were precipitated with 10% TCA, redissolved in Laemmli sample buffer and investigated by immunoblotting. Fractions containing RA33 were pooled, precipitated with ammonium sulphate, and redisolved in either 20 mM Tris-HCl, pH 7.5, containing 6 M urea, or trypsin sequencing buffer (see below).

Protein sequencing. 20 μg of purified RA33 were mixed with 0.1 ml of 125 mM ammonium bicarbonate, pH 7.5, containing 2 μg trypsin sequencing grade (Boehringer Mannheim GmbH, Mannheim, Germany) and incubated for 6 h at 37°C; an extra 2 μg of trypsin was added and incubation was continued for another 16 h. The resulting peptide fragments were separated by reversed phase HPLC on a 5 μm C18 column (Deltapak; Waters Associates, Millipore Corp., Milford, MA) using a linear 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid. Five fractions corresponding to major peaks on the chromatogram were analyzed on a pulsed liquid phase sequenator (Type 477; Applied Biosystems, Inc., Foster City, CA).

ssDNA chromatography of hnRNP particles. HnRNP particles were extracted from intact nuclei as described by Beyer et al. (17). Briefly, nuclei were resuspended in STM (saline-tris-magnesium) buffer (10 mM Tris-HCl, 2.5 mM MgCl₂, 0.1 M NaCl) at pH 7.0 and gently stirred for 30 min on ice. Nuclei were then pelleted at 700 g for 15 min, resuspended in STM, pH 8, and stirred on ice for another 30 min; this step was repeated twice. The pH 8 extracts were used for ssDNA chromatography (16): 4.5 ml of extract (10 mg protein) was loaded onto a 10 ml ssDNA agarose column (Bethesda Research Laboratories, Bethesda, MD) equilibrated with 50 mM phosphate buffer, pH 7.4, containing 0.1 M NaCl. After extensive washing of the column with the same buffer, bound protein was eluted stepwise with 0.2 M NaCl, 0.3 M NaCl, 0.5 M NaCl, and 2 M NaCl, all in 50 mM phosphate buffer, pH 7.4. Aliquots of protein containing fractions were precipitated with 10% TCA and analyzed by SDS-PAGE and immunoblotting.

Preparation of 40S hnRNP complexes. HeLa cells (Wisconsin) were grown in monolayers (175 cm²) in DME supplemented with 10% FCS and glutamine. Nucleoplasm was prepared from isolated nuclei by sonication in STM buffer, pH 7.4, essentially as described (16), except that Triton X-100 was omitted during sonication and all subsequent steps. Volumes were adjusted so that six monolayers yielded 1 ml nucleoplasm. To prepare 40S hnRNP complexes, 0.5 ml of nucleoplasm were treated with 4 or 8 U/ml micrococcal nuclease (P-L Biochemicals, Inc., Milwaukee, WI) in the presence of 5 mM CaCl₂ for 15 min at 30°C. The nuclease reaction was stopped by addition of EGTA to 20 mM. The nucleoplasm was then layered onto a 12-ml 10–30% sucrose gradient in RSB-100 and centrifuged at 4°C for 14 h at 28,000 rpm in a rotor (SW40; Beckman Instruments, Fullerton, CA). For reference, dissociated 70S E. coli ribosomes were run in parallel. Gradients were fractionated from bottom to top into 0.7 ml fractions and their absorbance at 260 nm was determined. Protein was recovered from gradient fractions by TCA precipitation and was analyzed by immunoblotting with patient serum.

Gel electrophoresis and immunoblotting. Samples were usually analyzed on 12% SDS-polyacrylamide minigels (8 × 5 cm × 0.75 mm); in some experiments electrophoresis was performed on 20 × 14 cm × 1.5 mm gels. Prestained protein markers from Bethesda Research Laboratories were used as mol wt references. Electrophoretic transfer to nitrocellulose (BA53; Schleicher and Schuell, Germany) was performed for 40 min at 250 mA for minigels, and for 2 h at 400 mA for large gels. For immunodetection, the nitrocellulose was blocked for 30 min with a solution of 3% nonfat dried milk in PBS, pH 7.4. Subsequently, the blots were incubated for 40 min with serum diluted 1:25 in the same buffer, washed 3 × 5 min with PBS containing 0.1% Triton X-100 (PBS-Triton) and incubated for 30 min with an alkaline phosphatase coupled anti-human IgG (Fc) 2nd antibody (Accurate Chemical & Scientific Corp., Westbury, NY) diluted 1:2,500 in PBS-Triton, 3% nonfat dried milk. Finally, bound antibody was detected as described (6).

Results

Purification of RA33. A HeLa nuclear extract containing ~200 mg protein was chromatographed on heparin-Sepharose as described in Methods. Immunoblot analysis showed that RA33 was detected exclusively in three fractions of the 1 M NaCl/6 M urea eluate (Fig. 1); as these fractions contained about 10 mg protein, RA33 was ~20-fold enriched. In addition to RA33, two proteins with estimated molecular masses measuring 36 and 37 kD migrating slightly above RA33 were reactive with the serum used for immunodetection of RA33 (Fig. 2 B, lane H-S). These proteins were not only reactive with the RA33 specific serum used in this experiment, but also with most anti-RA33 positive sera tested in addition (not shown) indicating an immunological relationship between these three antigens. The fractions were combined, precipitated with ammonium sulphate, redissolved and further separated by chromatofocusing. Approximately 80% of the total protein eluted in the flow-through of the PBE-94 column and in the initial fractions of the pH gradient (not shown). RA33 eluted in several fractions below pH 8; the first two of these fractions contained...
tained a > 90% pure preparation of RA33 as judged by Coo-
masse blue staining (Fig. 2 A, lane CHF) and immunoblotting
(Fig. 2 B, lane CHF) of the purified proteins following SDS-
PAGE. The two immunoreactive proteins copurifying on hepa-
rin-Sepharose were not completely separated from RA33 by
chromatofocusing, although a major portion of these two pro-
teins eluted prior to RA33 at more basic pH.

Sequence analysis of five tryptic peptides. Attempts to se-
quence the amino terminal part of purified RA33 failed, pre-
sumably because of a blocked NH terminus. The RA33 prepa-
ration was therefore digested with trypsin, and the resulting
fractions were separated by reversed phase HPLC. Five pep-
tides corresponding to major peaks of the HPLC chromato-
gram (not shown) were sequenced. Computer analysis re-
vealed that their amino acid sequences were 100% identical
with sequences of two proteins of the 40S hnRNP complex: the
core proteins A2 and B1 which have identical sequences except
for a 12-amino acid insert near the NH2-terminus of B1 (21).

Fig. 3 shows the sequence of A2 and the positions of the
sequenced peptides, labeled A-E. Thus, peptides A (Asn-
Tyr-Tyr-Glu-Gln-Trp-Gly-Lys) and B (Gly-Phe-Gly-Phe-Val-
Thr-Phe-Asp-Asp-His-Pro-Val-Asp-Lys) corresponded to
amino acids 27–34 and 142–156 of A2, respectively. These
residues are located in the evolutionarily conserved amino ter-
mal parts of the molecule (CS-RBD I and II), with peptide B
containing seven amino acids of the most highly conserved
octapeptide region, the RNP consensus sequence (Fig. 3). Pe-
ptides C, D and E (10, 15, and 20 amino acids, respectively)
corresponded to A2 sequences situated outside the conserved
region, and were therefore unique for this protein. In sum-
mary, the sequences of five peptides (68 amino acids corre-
sponding to 20% of the A2 sequence) showed 100% identity
with sequences of the A2/B1 protein of the hnRNP complex.
Therefore, it was concluded that the protein sequenced was
identical to A2 or B1.

RA33 binds to ssDNA agarose and is contained in the 40S
hnRNP particle. On the basis of the sequencing data alone, it
was not possible to distinguish between A2 and B1. Moreover,
the possibility could not be excluded that the RA33 prepa-
ration used for sequencing was a mixture of RA33 and A2/B1.
In other words, RA33 and A2/B1 could have been copurifying
proteins migrating in SDS-PAGE as a single band. Therefore, it
was necessary to demonstrate that RA33 was present in the
hnRNP complex and exhibited the same properties as either
A2 or B1.

As most proteins of the hnRNP complex bind with inter-
mediate or high affinity to RNA or ssDNA (16, 25), binding of
RA33 to ssDNA was investigated by fractionating a nuclear
extract on ssDNA agarose as described in Methods. Bound
proteins were eluted by gradually increasing the NaCl concen-
tration from 0.1 to 2 M (Fig. 4 A). With the exception of the C
proteins, the hnRNP proteins are known to coelute from
ssDNA agarose between 0.3 and 0.5 M NaCl (16). Most of the
proteins were collected in the flow-through of the column (0.1
M NaCl), and several weakly stained bands can be seen in the
0.2 and 0.3 M NaCl eluates. In the 0.5 M fraction, however,
a dominant double band and a faint doublet slightly above are
clearly visible in the 30–40 kD region: this pattern of doublet
bands is similar to the characteristic pattern described for the
A and B proteins which in oligomeric hnRNP complexes are
present in the molar ratio 3A1–3A2:1B1–1B2 (17, 26). The lower
band of the dominant doublet was identified to be A1 by the A1
specific monoclonal antibody 4B10 (16) (not shown) and an
A1 specific patient serum (Fig. 4 B, lanes B). The upper band
corresponding in size to A2 was immunostained by an RA33
specific serum (Fig. 4 B, lanes A). This serum also stained the
two minor bands above that most probably contained the B
proteins. Interestingly, only trace amounts of other proteins
were present in this fraction, whereas the 2 M eluate contained
at least 20 different proteins.

In a second experiment, 40S hnRNP particles were isolated
from a nuclear extract by sucrose gradient centrifugation. This
procedure yielded a preparation of highly enriched hnRNP
core proteins (17, 26). Subsequently, the presence of RA33
was detected using the RA33 reference serum, which predomi-
nantly stained a band in the mol wt region of the A proteins.
the positions of the hnRNP proteins A1 and A2 in panel A, and of the autoantigens A1 (visible as double band; see also 17, 27) and RA33 in (B) are indicated by arrows.

Thus, it was demonstrated by independent experimental approaches that RA33 was indistinguishable from the hnRNP core protein A2. The two immunoreactive proteins copurify with RA33 in ssDNA chromatography could not be identified unambiguously. However, their migration relative to A1 and A2 on SDS gels appeared similar to that reported for the B1 and B2 proteins of the hnRNP particle.

**Frequency and diagnostic specificity of anti-RA33 (anti-A2).** Purified RA33 was used as antigenic source on Western blots to show that the antigenic properties of the purified material were comparable to that contained in crude nuclear extracts. 15 RA33 positive sera were retested and found to be reactive also with highly purified RA33 (not shown). In addition, a total number of 120 sera from patients with RA, SLE, mixed connective tissue disease (MCTD), Sjögren’s syndrome, polymyositis, scleroderma, reactive arthritis, osteoarthritis, and from healthy controls were investigated using a RA33 preparation partially purified by heparin-Sepharose chromatography (Table I). Taken together, antibodies to semipuri-

**Discussion**

The data presented here reveal that the previously described nuclear autoantigen RA33 is indistinguishable from the A2 hnRNP protein, one of the six core proteins of the hnRNP complex (17, 19, 21). Evidence for this is based on the following observations: (a) the amino acid sequences of five tryptic peptides obtained from a highly purified RA33 preparation can be found in that of the A2 protein; (b) the chromatographic behavior of RA33 was indistinguishable from A2 in ssDNA affinity chromatography; (c) RA33 was contained in the 40S hnRNP particle isolated by sucrose gradient centrifugation; and (d) the molecular masses (estimated from SDS-PAGE) reported for RA33 and A2 are very similar, namely 33 kD for RA33 (6) and 32.5–36 kD for A2 (16, 17, 21, 27). The sequencing data are also compatible with RA33 being the hnRNP core protein B1 which is identical to A2 except for a 12-amino acid insertion near the NH₂ terminus (21). How-

**Figure 4.** ssDNA chromatography of a low salt HeLa cell nuclear extract (NET). Proteins eluting at the NaCl concentrations indicated were analyzed by SDS-PAGE followed by Coomassie blue staining (A) or immunoblotting (B). The immunoblot was probed with the RA33 specific serum (lanes A) and with an A1 hnRNP specific patient serum (lanes B). The positions of the hnRNP proteins A1 and A2 in panel A, and of the autoantigens A1 (visible as double band; see also 17, 27) and RA33 in (B) are indicated by arrows.

**Figure 5.** Analysis of the 40S hnRNP complex by immunoblotting. Fractions derived from sucrose gradient sedimentation of HeLa cell nucleoplasm (NPL) were analyzed by immunoblotting for the presence of RA33. Top, bottom, and the position of the 30S-40S region of the gradient are indicated. Gradient fractions 1–8 were electrophoresed separately, fractions 9/10, 11/12, 13/14, 15/16 and 17/18 were combined. H-S designates the partially purified RA33 preparation obtained by Heparin-Sepharose chromatography which was used as positive control. Arrows indicate the position of RA33 in the NPL and the H-S lane.

| Table I. Frequency of Autoantibodies to Purified RA33 (A2 hnRNP) in Inflammatory and Degenerative Rheumatic Diseases |
|---------------------------------|----------|--------|-----|
| Patients                        | n        | Positive sera | %   |
| RA                              | 30       | 10      | 33% |
| SLE                             | 10       | 2       | 20% |
| MCTD                            | 10       | 4       | 40% |
| Sjögren’s syndrome              | 5        | 0       |     |
| Polymyositis                    | 5        | 0       |     |
| Scleroderma                     | 5        | 0       |     |
| Reactive arthritis              | 20       | 0       |     |
| Osteoarthritis                  | 15       | 0       |     |
| Healthy controls                | 20       | 0       |     |
ever, experimental evidence suggests that B1 corresponds to the relatively faint 36 kD band that copurified with RA33 in chromatofocusing (Fig. 2): The estimated molecular mass of this protein is similar to that reported for B1, which ranges between 35.5 and 38 kD (16, 17, 21, 27). Next, the staining of the RA33 band and the 36–37 kD doublet (Fig. 2, lane H-S) indicated that RA33 was present in severalfold excess over these two proteins. This is compatible with the assumed molar ratio of 3:1 for A2 to B1 (and B2) in the hnRNP complex (26). Finally, when the A/B core proteins were isolated by ssDNA chromatography (a method that can be used for partial purification of hnRNP proteins [16]), the RA33 serum reacted with three antigens corresponding in size to A2, B1 and B2. These antigens were apparently identical to the three antigens copurifying in heparin-Sepharose chromatography and (partially) in chromatofocusing. This interpretation is also compatible with the reported immunocrossreactivity of anti-A2 to B1 and B2: mouse antibodies affinity-purified from recombinant A2 bound not only to A2 but also to both B proteins (21). Given the high degree of sequence homology between A2 and B1, reactivity of both proteins with the same (auto) antibody would not be surprising. In fact, most RA33 positive sera have been found to be reactive also with the 36- and 37-kD antigens assumed to be B1 and B2 (28, 29, unpublished observation).

In this context, it should also be mentioned that monoclonal antibodies raised against hnRNP proteins frequently recognize several different proteins (13, 30).

When the A/B proteins were isolated by ssDNA chromatography anti-RA33 antibodies clearly reacted with the A2 band (and also with both the B1 and B2 band), but not with the A1 band. This was important to demonstrate since (recombinant) A1 has already been identified as an autoantigen in a variety of connective tissue diseases (14). Even though A1 and A2 are related proteins sharing extensive sequence homologies in their NH2-terminal portions (21), anti-RA33 (A2) positive sera reacted only very infrequently with A1 (unpublished observation).

Since, however, their COOH-terminal parts are only distantly related one could speculate that the COOH-terminal part of A2 contains the epitope(s) eliciting A2 specific immune responses.

So far, hnRNPs have been studied only occasionally as target structures for autoantibodies (11, 12). In a study by Gelpi et al. (13), antibodies against hnRNPs were detected with high frequency in anti-U1RNP positive sera. As these sera reacted with one or more of the hnRNP proteins no preferential targets for the reacting autoantibodies could be identified. More recently, antibodies to recombinant A1 hnRNP were described in patients with various connective tissue diseases (14). In contrast, a high disease specificity has been described for RA33 as antibodies against this protein were found almost exclusively in RA patients (6). This high specificity was not fully maintained when purified RA33 (i.e., A2), was used as antigen in immunoblotting since anti-RA33 antibodies were also detected in 4 of 10 sera from patients with MCTD and in 2 of 10 SLE sera (Table 1). Interestingly, these 6 sera contained antibodies to U1snRNP in addition to anti-RA33; this could indicate the existence of crossreacting epitopes. The diagnostic value of anti-RA33 (anti-A2) is not severely impaired by this result since RA sera neither contain antibodies to U1snRNP nor other specificities typically found in SLE sera (anti-DNA, anti-Sm, anti-Ro, anti-La). Nevertheless, the clinical significance of anti-RA33 in MCTD and SLE, as well as the possible crossreactivity of anti-RA33 and U1snRNP, remains to be determined and will be the subject of future studies. Most importantly, all sera from patients with reactive arthritis and osteoarthritis were found negative for anti-RA33. Thus, this autoantibody is extremely useful to serologically differentiate RA from other arthritic diseases, which is often difficult, especially in the early stages of these diseases.

Although the detailed function of the hnRNP core proteins is not fully understood, they are known to closely interact with heterogeneous nuclear RNA (17, 31, 32). At least the C proteins of the hnRNP complex have been shown to be involved in processing of pre-mRNA, thus demonstrating a functional and topographic link between hnRNPs and snRNPs (24, 33–36). Since monoclonal antibodies against A2 are not yet available purified, human autoantibodies could be used as scientific tools to further investigate structure and function of the hnRNP complex.

In summary, we have demonstrated that RA33 is indistinguishable from the A2 hnRNP protein, thereby identifying another ribonucleoprotein as an important autoantigen in rheumatic diseases. Subsequent studies using deletion mutants of recombinant A2 and linear peptides will elucidate the immunologically relevant regions of this important autoantigen. This could give additional insights into the pathogenetic mechanisms of rheumatoid arthritis and thus deepen our understanding of this autoimmune disorder.

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