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Cytoplasmic detection of a novel protein containing a nuclear localization sequence by human autoantibodies

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SUMMARY

A great diversity of antibodies directed to cell proteins has been described in sera of patients with autoimmune diseases. Most of these sera recognize nuclear components, but some others are directed against cytoplasmic autoantigens. Some of the antibodies directed to cytoplasmic autoantigens are well characterized, such as anti-mitochrondial, anti-ribosomal, anti-microsomal and anti-Golgi complex autoantibodies, but the target of many others remains unknown. In the last 5 years we have selected 32 sera with a characteristic speckled cytoplasmic pattern in indirect immunofluorescence (IIF) assay among a total of more than 31 000 sera from patients with any kind of autoimmune manifestation who attend our Connective Tissue Disease Clinic. Using a human cDNA expression library, we have identified a new autoantibody specificity named RCD-8 in five of these sera, directed to one cytoplasmic autoantigen. Affinity-purified antibodies eluted from a positive clone reproduced the same IIF cytoplasmic staining pattern as native serum and reacted with one single band of 160 kD on an immunoblot of HeLa cell extract. The sequence was found homologous to an autoantigen recently reported named Ge-1, and contains a nuclear localization sequence (NLS), an active protein domain made by a contiguous stretch of amino acids which allows the selective entry of the protein into the nucleus. The five patients whose sera exhibited this new autoantibody specificity displayed different autoimmune pathological profiles.

Keywords autoantibodies nuclear localization sequence cDNA human expression library

INTRODUCTION

A characteristic feature of autoimmune diseases is the presence of autoantibodies directed against many nuclear and cytoplasmic cell components [1]. Most autoantigens are intranuclear and are involved in essential or important cell functions, such as pre-mRNA splicing, DNA replication and transcription. Autoantibodies directed against precise cytoplasmic components such as mitochondria, ribosomes and Golgi complex have been described [2], but the antigenic target of many other anti-cytoplasmic autoantibodies remains unknown. As a good correlation between certain autoantibodies and some clinical syndromes has been demonstrated, the presence of certain autoantibodies has provided valuable help in clinical diagnosis of many autoimmune diseases [3]. Moreover, the characterization of the autoantigens recognized by autoimmune sera might help to improve our knowledge of the etiological and pathogenic mechanisms that induce autoimmunity [4].

Screening of an expression cDNA library has proved a useful method for description of new autoantigens. Autoantibodies from patients with diverse autoimmune diseases have been used in the clonal isolation and characterization of many autoantigens [2,5]. In order to investigate the fine structures recognized by autoantibodies exhibiting uncommon cytoplasmic immunofluorescence staining, we used a HeLa cDNA library cloned in a λZAP II expression vector, and found a new cytoplasmic autoantibody specificity present in the sera of five patients suffering from different autoimmune diseases.

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MATERIALS AND METHODS

Human sera

During the last 5 years more than 31 000 human sera have been studied at our laboratory in order to investigate the presence of autoantibodies. Excluding anti-mitochondrial, anti-microsomal and anti-ribosomal well defined patterns, 32 sera showed a speckled cytoplasmic pattern when tested by indirect immunofluorescence (IIF) on rat tissues (liver, kidney, stomach) and/or HEp-2 cells.

Cell lines

HeLa and HEp-2 cells (ATCC CCL 2.2 and 23; American Type

Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Sera-Lab, Sussex, UK), 2 mM L-glutamine and 5 μg/ml gentamicin sulphate in a 5% CO_2/95% air incubator.

Indirect immunofluorescence

Commercially available HEp-2 cell line preparations (Kallestad Labs, Chaska, MN) were used as substrate. Primary antibody was incubated on slides for 1 h in a humid atmosphere at room temperature and washed extensively with PBS to remove any unbound antibody. Bound antibodies were detected with FITC-conjugated rabbit anti-human IgG (Dako, Carpinteria, CA). After washing, the slides were read on an epifluorescence microscope.

Ouchterlony double immunodiffusion

Commercially available agarose plates (MarDx, Carlsbad, CA) were used. Primary antibody was tested against rabbit thymus extract by using reference sera. Plates were incubated at room temperature for a minimum of 72 h and then washed with 5% (w/v) sodium citrate to remove any non-specific precipitation.

SDS–PAGE and immunoblotting

Immunoblot analysis techniques were carried out by using cell extracts derived from HeLa and HEp-2 cells. Briefly, 50×10^6 exponentially growing cells were lysed in 250 μl of NET-2F (50 mM Tris–HCl pH 7·4/150 mM NaCl/5 mM EDTA/0·1% SDS/0·5% Nonidet P-40/0·5% sodium deoxicholate/0·02% NaN_3, 1 mM phenylmethylsulfonyl fluoride/0·1% iodoacetamide) for 20 min at 4°C and centrifuged at 12 000 ***g*** for 10 min at 4°C to eliminate cell debris. Electrophoresis of cell lysates was performed in a 10% acrylamide gel as described by Laemmli [6]. Proteins were transferred to nitrocellulose as described by Towbin *et al*. [7]. After transfer, nitrocellulose strips were blocked with 3% (w/v) non-fat dry milk in TBS–T (10 mM Tris–HCl pH 7·5/150 mM NaCl/0·05%/Tween-20) for 1 h at room temperature. Primary antibody was then incubated with the strips for 1 h at room temperature and washed extensively with TBS–T to remove any unbound antibody. Bound antibodies were detected by incubating the nitrocellulose strips with alkaline biotin-conjugated rabbit anti-human IgG and alkaline phosphatase (AP)-conjugated streptavidin (Dako). After washing, the membrane was developed with NBT/BCIP (Boehringer, Mannheim, Germany) in AP buffer (100 mM Tris–HCl pH 9·6/100 mM NaCl/5 mM $MgCl_2$).

cDNA library screening

A HeLa (D98/AH-2, $HPRT^-$ subclone) cDNA library cloned in λZAP II expression vector was obtained from Stratagene Inc. (La Jolla, CA). Clones were initially selected by immunological screening as described by Young & Davis [8]. The test was performed on duplicate filters and those phages reacting with antisera were subsequently purified to 100%. Before screening, the serum was extensively adsorbed against bacteria and wild-type λZAP II phage to eliminate background. Bound antibodies were detected by immunoblot as described above.

Affinity purification of autoantibodies

Affinity purification of antibodies from λZAP II clones was performed using confluent plates induced to produce recombinant protein with isopropyl-β-D-thiogalactopyranoside (IPTG)-impregnated nitrocellulose filters. After overnight incubation, the filters were blocked, probed with primary antibody, and washed, as described for immunoblot analysis. Bound antibody was eluted from the filters with 5 ml 0·1 M glycine buffer pH 2·5–2·8 by rocking at room temperature for 5 min and neutralized with 250 μl of 1 M Tris–HCl pH 9·5. Eluted antibodies were concentrated in Centricon 30 microconcentrators (Amicon Corp., Canvers, MA) before they were used for IIF and immunoblot assays.

DNA subcloning and sequence determination

Purified λZAP II clones were subcloned *in vivo* into pBluescript SK- plasmids by using R408 helper phage as recommended by the manufacturer (Stratagene). DNA sequencing was carried out according to the dideoxy technique of Sanger *et al*. [9] by using biotinylated synthetic oligonucleotide primers. After electrophoresis, DNA was transferred to positively charged nylon membrane for capillary action and immobilized on the membrane by UV irradiation. The nucleotide sequence was detected by the SEQ-LIGHT chemiluminescent DNA sequencing system as recommended by the manufacturer (Tropix Inc., Bedford, MA). Computer analysis of nucleic acid and protein sequences was done using the University of Wisconsin Genetics Group Sequence Analysis Software Package [10].

Immunoblot from recombinant protein

A single bacterial colony expressing the recombinant protein was transferred into 5 ml LB medium containing ampicillin (100 μg/ml), grown overnight at 37°C with vigorous shaking and induced to produce the protein with 10 mM IPTG for 1 h. Bacteria were harvested by centrifugation, resuspended in 1 ml of 50 mM glucose/10 mM EDTA/25 mM Tris–HCl pH 8·0, containing 4 mg/ml lysozyme and incubated at room temperature for 5 min. After centrifugation, the spheroplasts were resuspended in 500 μl NET-2F lysis buffer prechilled to 4°C, incubated for 30 min on ice with occasional mixing and centrifuged at 12 000 ***g*** for 10 min at 4°C to eliminate debris. Electrophoresis of bacterial lysates, transfer of proteins and immunological detection were performed as described above.

Mapping of the immunoreactive region

Fragments of RCD-8 cDNA clone were prepared by treatment with restriction enzymes or with exonuclease III by using the Erase-a-Base kit (Promega Biotech, Madison, WI), and ligated into pGEX expression vectors (Pharmacia, Piscataway, NJ). DNA sequence analysis of the mutants confirmed the structure predicted by the constructed complementary DNA clones. Expression of fusion proteins and immunoblot analysis were performed as described above. Successful production of GST-RCD-8 fragment fusion proteins was confirmed by immunoblot analysis using a goat anti-GST antiserum (Pharmacia).

RESULTS

Selection and characterization of sera

RCD serum was selected as a prototype for screening a HeLa λ ZAP II expression library because it showed the characteristic cytoplasmic scattered speckled staining and a complete lack of nuclear staining (Fig. 1), and in immunoblotting using HeLa and HEp-2 cell line extracts only two bands of 160 and 52 kD were obtained. No differences in molecular weight of the bands in both cell lines were observed (Fig. 2).

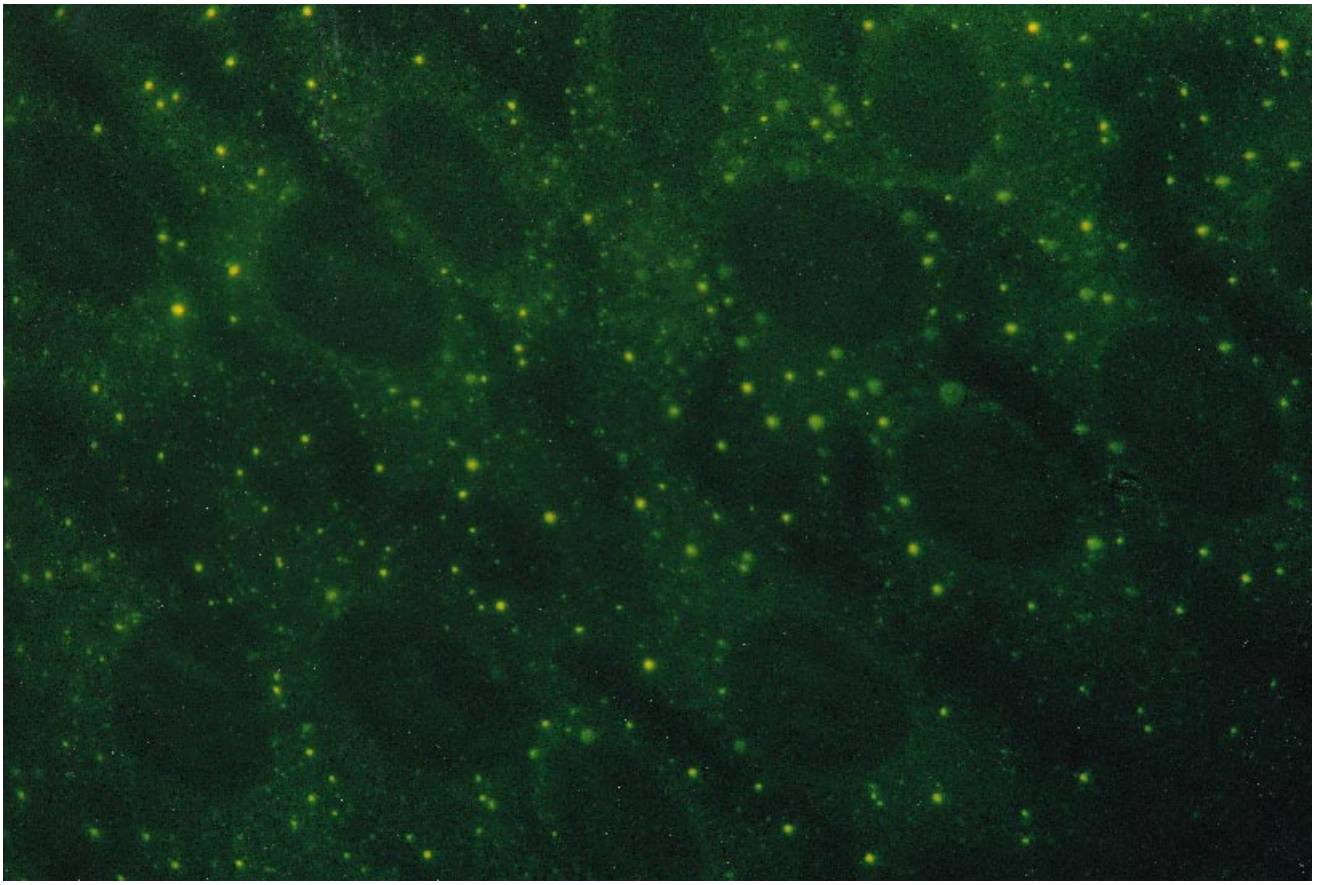

Fig. 1. Indirect immunofluorescence **(**IIF) of RCD serum on HEp-2 cells showing a cytoplasmic scattered speckled staining.

cDNA library screening

After screening 500 000 plaques, one positive signal was obtained, RCD-8, which retained specificity after a secondary and tertiary screening to 100% purity. Antibodies from the RCD serum were purified on nitrocellulose filters containing 50 000 phages expressing the recombinant protein. Once eluted and concentrated, the affinity-purified antibodies were tested by immunoblot on electrophoresed HeLa cell line extract and IIF. On IIF assay, the antibodies eluted from clone RCD-8 showed a similar staining pattern as that observed with RCD serum. By immunoblotting they recognized a single 160-kD band (Fig. 3), one of the two bands obtained with native RCD serum.

cDNA sequencing

cDNA from clone RCD-8 was subcloned *in vivo* into pBluescript SK- plasmid and a single insert of 3400 bp was observed. The nucleotide sequence of clone RCD-8 was determined from both strands. The complete sequence of 3332 nucleotoide was submitted to GenBank (accession number U17474). Within this sequence there is an open reading frame beginning with the first ATG at nucleotide 77 and ending with a TAG at nucleotide 2980. The coding region is followed by a 3′ untranslated region containing a putative polyadenylation signal (ATTAAA) at nucleotide 3312 [11]. This sequence encodes a 968-amino acid polypeptide (Fig. 4) with a predicted molecular mass of 103 828 D. There was a difference between the predicted molecular mass of 103·8 kD for the protein encoded by this clone and the molecular mass of 160 kD observed in immunoblot of HeLa cell extract.

The nucleotide and deduced amino acid sequences obtained were checked with the GeneBank and the EMBL data banks for homologous sequences. This sequence was found to be 99·9% homologous from nucleotides 1370 to 4659 to the nucleotide sequence of autoantigen Ge-1 recently reported by Bloch *et al.* [12], except for the changes despicted in Fig. 4. The most important change is the insertion of one nucleotide (G) at position 2341 on RCD-8, which produces a variation in the reading frame. As a consequence, RCD-8 is 186 amino acids longer than Ge-1 at the C-terminal end, Ge-1 being 433 amino acids longer than RCD-8 at the N-terminal end.

Screening of sera

To investigate the presence of anti-RCD-8 in the rest of the sera with the characteristic cytoplasmic pattern, 31 sera were absorbed with bacterial proteins and tested by immunoblotting against bacterial cell extract containing IPTG-induced RCD-8 recombinant protein. Four sera, OBB, IAM, CPL and MRB recognized RCD-8 recombinant protein (data not shown). These sera revealed the same cytoplasmic pattern as RCD serum, and, with the exception of CPL, none of them showed antinuclear antibodies. By immunoblot they revealed, in addition to others, the 160-kD band. Antibodies from each serum were purified by using RCD-8 clone and then tested by IIF and immunoblot on electrophoresed

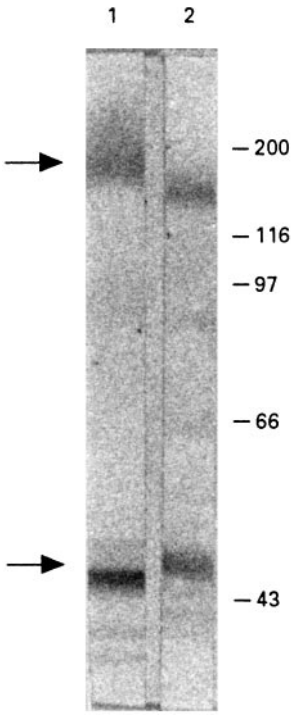

Fig. 2. Immunoblot analysis with serum RCD of HeLa (lane 1) and HEp-2 (lane 2) cell extracts. Two bands of 160 and 52 kD are detected (arrows). No differences in the molecular mass of the bands from both cell lines were observed. Positions of molecular mass markers are shown on the right in kD.

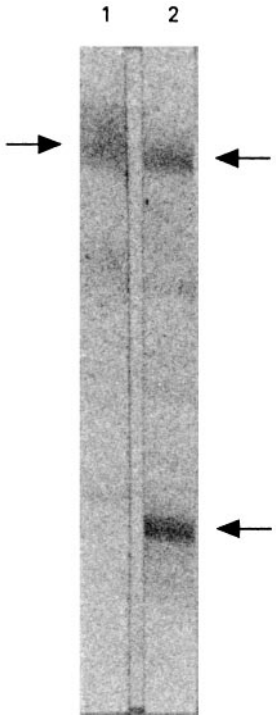

Fig. 3. Immunoblot analyis of HeLa cell extract with affinity-purified antibodies from clone RCD-8 (lane 1) and native serum (lane 2). Affinity-purified antibodies show a single 160-kD band (arrow).

HeLa cell extract. In all cases the results revealed the same IIF staining pattern, and by immunoblot, a single 160-kD band. Antibodies purified from CPL serum did not show any nuclear staining.

Mapping the immunoreactive region in RCD-8

Autoantibodies purified from the five positive sera reacted with fusion proteins containing amino acids 1–735 of RCD-8 (Fig. 5, lane 3), but did not react with fusion proteins containing amino acids 1–435 (lane 2), 1–354 (lane 4), 500–968 (lane 5). The presence of recombinant protein in each lane was confirmed by incubating an identical filter with goat anti-GST antiserum (data not shown). These results demonstrated that RCD-8 autoantibodies present in RCD, OBB, IAM, CPL and MRB sera recognized an immunoreactive region localized between amino acids 435 and 500.

DISCUSSION

In this study we have investigated the molecular basis of specificity recognized by some sera from patients with different autoimmune disorders displaying a well characterized but uncommon pattern of cytoplasmic scattered speckles staining in an immunofluorescence assay. From a total of 32 sera with this cytoplasmic staining pattern, we selected a serum (RCD) for screening a human cDNA expression library because of its lack of antinuclear activity and because of its restricted reactivity with two bands of 160 and 52 kD on an immunoblot of HeLa cell extract. By using this serum for the screening of a HeLa λ ZAP II expression library, a cDNA clone, RCD-8, was isolated. Following a strategy used by us for the description of anti-RPA autoantibodies [5], we obtained affinity-purified antibodies eluted from the positive clone. These antibodies reacted with one single band of 160 kD on an immunoblot assay using a HeLa cell extract and, in IIF assay, reproduced the cytoplasmic staining pattern obtained with native RCD serum. Four other sera from the 32 investigated displayed anti-RCD-8 recombinant protein reactivity.

After clone cDNA sequencing, a discrepancy was observed between the molecular mass predicted of 103·8 kD and the 160-kD band detected by immunoblot, suggesting that RCD-8 cDNA encoded only a part of the whole protein and the actual autoantigenic protein is not yet defined. Precedent for this possibility is provided by the analysis of the relationship between RCD-8 and Ge-1. When genetic data banks were examined, a nearly 100% homology was found with the 3′ end of an autoantigen cDNA recently sequenced, named Ge-1 [12]. The most important difference between Ge-1 and RCD-8 is the addition of a nucleotide (G) at position 2341 of RCD-8 which causes a change in the reading frame. As a consequence, the deduced sequences of Ge-1 and RCD-8 are different in the C-terminal end from this position. Although differences between both sequences could be due to mutations in the cloned cDNA, the possibility exits that there are two different forms of the protein, since it occurs with the 60-kD SS-A/Ro autoantigen, from which two sequences, named 60α [13] and 60β [14], have been reported and where the only difference lies in the C-terminal residues. Alternatively, Ge-1, RCD-8 and the present unknown antigen each differ from one another by a frameship which could have come from the same gene by alternative splicing.

Ge-1 autoantigen was described by Bloch *et al.* [12] as a nuclear protein, based on two facts. First, it possessed an active nuclear

Fig. 4. Amino acid sequence of RCD-8 and Ge-1. Antigenic region is in bold characters. NLS region is underlined.

localization sequence (NLS), a protein motif allowing protein recognition by other specific proteins named NLS-binding protein (NBP), which, in turn, transport the NLS-containing proteins into the nucleus [15]; and also, because sera obtained from rats immunized with Ge-1 recombinant protein showed, in an IIF assay, a speckled nuclear but not cytoplasmic pattern. However, native GE-1 serum exhibited a homogeneous nuclear and a speckled cytoplasmic staining pattern. The present work, based on the use of human antibodies purified after elution with the recombinant protein, seems to indicate that RCD-8 is localized in the cytoplasm. Furthermore, four out of the five autoimmune sera positive with the recombinant protein on immunoblot did not show any antinuclear reactivity in IIF.

Nuclear protein import is a selective and regulated process. Proteins destined for the nucleus contain NLS, but their transport from the cytoplasm to the nucleus occurs only at a certain stage of the cell cycle or in response to diverse factors [15]. Even if, in the present state of knowledge, the function of RCD-8 protein is still unknown, the explanation for our finding could be related to the protein function and its intracellular cycle. Hence, the only unanticipated finding is the nuclear staining of rat anti-Ge-1 protein antibodies, although it has been extensively demonstrated that epitopes recognized by human autoantibodies are different from those recognized by sera from immunized animals. On the other hand, the antibodies present in all our five autoimmune anti-RCD-8 sera as well as in Ge-1 serum react with the same antigenic region of 65 amino acids, which contains the NLS region (from amino acids 477 to 494 of RCD-8). It is possible that, in the cytoplasm, that region might be exposed and hidden at the nucleus following protein conformational changes.

From a practical point of view, as observed in other autoantibodies such as anti-Alu RNA protein, anti-high mobility group proteins, etc. [16], it was not possible to relate the presence of antibodies anti-RCD-8 with a defined autoimmune disorder. RCD serum was collected from a woman with scleroderma by the criteria of the ARA [17] and secondary Sjögren's syndrome (SS) by the criteria of the European Community Study Group on diagnostic criteria for SS [18]. OBB serum was collected from a woman with rheumatoid arthritis by the criteria of the ARA [19] and sicca syndrome. CPL was obtained from a patient with systemic lupus erythematosus by the criteria of the ARA [20]. The clinical history of IAM was not available. Patient MRB presented with a cerebral vascular accident.

Finally, we wish to point out that in this work we were only able to define the nature of the antigen recognized by five out of 32 autoimmune sera showing uncommon anti-cytoplasmic patterns.

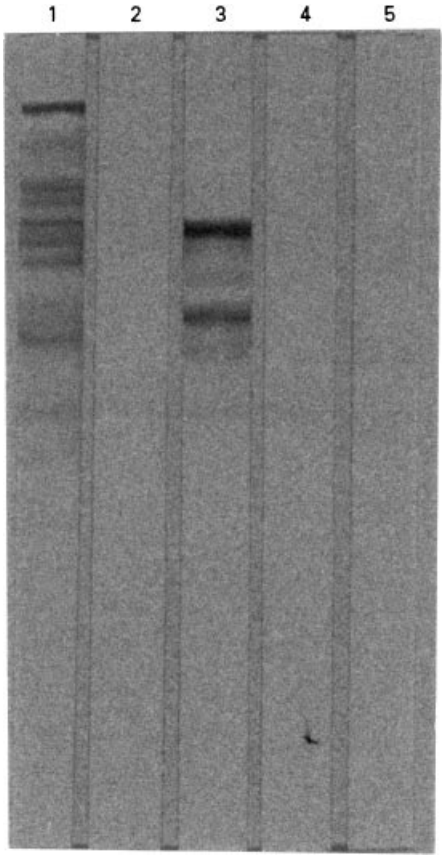

Fig. 5. Immunoblot analysis of autoantibodies purified from RCD serum using RCD-8 recombinant protein with fusion proteins containing amino acids of RCD-8: 1–435 (lane 2), 1–735 (lane 3), 1–354 (lane 4) and 500–968 (lane 5). Lane 1 shows the immunoblot analysis of these autoantibodies purified with the total recombinant protein. The additional bands observed in lanes 1 and 3 correspond to degradation products.

This suggests that, like nuclear autoantigens, the diversity of the autoantigens located in the cytoplasma seems also to be very wide.

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